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13. ABSTRACT (Maximum 200 Words)

This is an annual report of the 1st grant year. Since PI and Research Associates moved to the new Institute September 2003, the transfer of grantee institute is under processing.

We established chitin particle preparations from 4 different sources and selected one preparation that expresses the highest Th1 adjuvant activity. We completed the preparation at amounts necessary for this entire project.

Mycobacterial components, Th1 adjuvants, have been proposed to use for the down-regulation of allergic responses. However, our studies (Task 2) indicate that, unlike chitin Th1 adjuvant, mycobacterial components induce PGE2-releasing macrophages in the spleen and a Th1-to-Th2 shift of immune responses. Our results suggest that mycobacterial components are unreliable for long-term uses as a Th1 adjuvant.

14. SUBJECT TERMS

Childhood asthma, N-acetyl-D-glucosamine polymer, IL-12, GATA-3 T-bet, macrophages, airway hyperreactivity

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INTRODUCTION:

This annual report includes a brief summary of the 1st year research and related activities supported by DAMD17-03-1-0004.

This project funded by DOD DAMD17-03-1-0004 was initiated at East Carolina University on February 24, 2003. During the project year at East Carolina University, Drs. Hiroyoshi Ohata and Akihito Nishiyama, Research Associates, joined in June 2003. Ohata and Nishiyama have been interacting with Drs. Michael R. Van Scott, John F. Bradfield, and Ruth Ann Henriksen, Co-investigators/Consultants listed in the project, and establishing techniques necessary for animal experiments, molecular characterizations, and cytometric detections.

Since the Department of Biomedical Sciences, Florida Atlantic University (FAU), Boca Raton, FL, offered PI an Associate Professorship position with tenure and great supports for this DOD grant project, PI and research associates in his lab moved to FAU in September 2003. The last day of work at East Carolina University was September 29, 2003. Since September 30, 2003, Shibata (PI), Ohata and Nishiyama have started setting up the lab in FAU and preparing the grant transfer to FAU. Jon Gabbard, B.S., joined the lab as Lab Manager in November 2003. Setting up the lab is supported by FAU's start-up program.

On March 15, 2004, in order to request the grant transfer, PI submitted a complete set of revised proposal to Ms. Karen Stotler, karen.stotler@us.army.mil, Contract Specialist, U. S. Army Medical Research Acquisition Activity. The final decision of "Transfer" is pending.

BODY:

Task 1: To determine if oral administration of 1 – 4 μm particles of chitin will down-regulate airway hyperreactivity (AHR) and GATA-3 levels as a measure of Th2 responses, and enhance T-bet levels as a measure of Th1 responses in the lungs of mice that are sensitized with ragweed allergens.

a. Establish the effects of dose response of chitin particles (Months 1 – 4).

b. Establish therapeutic/prophylactic effects of chitin (Months 4 – 9).

c. Determine duration of chitin treatments (Months 8 - 12).

We prepared 4 different sources of 1 – 10 μm chitin preparations and measured their Th1 adjuvant activities. Based on the activities, we selected a reliable source of chitin. We completed the preparations of chitin particles (Chitin B) at amounts needed for this entire project (Table 1). Previous studies (1) indicating down-regulation of chitin treatment on airway allergic responses will be presented at the CDMRP's Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico.

Chitin is a naturally occurring N-acetyl-D-glucosamine polymer and new class of Th1 adjuvant that stimulates IL-12 production by macrophages *in vitro* (2,3). This study (1) demonstrates that chitin down-regulates the allergic response in a murine model of allergic asthma. Ragweed-sensitized BALB/c mice were treated orally with saline or 1- 10 μm chitin particles (8 mg/day for 3 days before and 13 days during ragweed allergen immunization, 7 mice per group). The mice were challenged with ragweed intratracheally on day 11. Three days after the challenge, serum IgE levels and lung eosinophil numbers were quantified. Th2 responses were further explored by measuring cytokine production by spleen cells isolated from the ragweed-immunized mice (controls) and cultured in the presence of ragweed and/or chitin for 3 days. The ragweed-sensitized mice treated with saline showed high levels of serum IgE and lung eosinophils, and splenocytes from these animals produced IL-4, IL-5, and IL-10 in vitro. Chitin treatment resulted in a significant reduction of these Th2 parameters ($p<0.01$). Collectively, these results indicate that chitin, which induces innate immune responses, down-regulates Th2-facilitated IgE production and lung eosinophilia in the allergic mouse. Oral administration of chitin therefore represents a potentially effective treatment for IgE-mediated allergic diseases, including childhood asthma. We will continue to define the mechanisms, the dose-responses, and the therapeutic/prophylactic effects of chitin treatment in the following years.

Task 2: To determine if the effects of 1 – 4 μm particles of chitin on endogenous IL-12- or IFNγ- mediated down-regulation of airway allergic responses will be greater than those of HK-BCG, ODN-CpG or an equal number of particles of 1 – 10 μm chitin.

a. Establish comparative studies on the effects of 1 – 4 μm chitin, 1 – 10 μm chitin, HK-BCG and ODN-CpG (Months 12 - 24).

b. Study if endogenous IL-12 or IFNγ is required for the chitin-induced down-regulation of GATA-3-mediated allergic responses (Months 24 – 40).

HK-BCG and ODN-CpG as well as chitin particles are well established Th1 adjuvants. However, unlike chitin particles (4), HK-BCG and ODN-CpG potentially induce PGE_2-releasing macrophage (PGE_2-MØ) in the spleen (4-9). We are establishing that splenic PGE_2-MØ may eventually down-regulate their Th1 adjuvant activities. Four abstracts and three manuscripts have been prepared. A summary of the finding is below.

Hosts develop Th1 immunity during the early stages of mycobacterial infections, but induce a switch to Th2 immunity during the later stage of infection. Mycobacterial products induce PGE_2-releasing macrophages (PGE_2-MØ) in the mouse spleen. Since PGE_2 inhibits Th1 responses with enhanced Th2 responses, we determined whether splenic PGE_2-MØ induce this immune shift. Heat-killed *Mycobacterium bovis* BCG at 0.01 and 1 mg/dose was given i.p. to Balb/c and C57B1/6 mice. Splenic PGE_2-MØ were characterized by the *ex vivo* release of PGE_2 (>1 ng/10^6 cells) and the expression of prostaglandin G/H synthase (PGHS) -1, PGHS-2, membrane PGE synthase (PGES) and cytosolic PGES. PGE_2-MØ were detected 14 days after treatment with 1

mg, but not with 0.01 mg, BCG. BCG-treated mice also expressed (i) serum IgG1 and IgE antibodies against mycobacterial antigens and (ii) IL-4 and IL-10 production by spleen cells stimulated *ex vivo* with mycobacterial antigens (recall responses). Treatment of BCG-injected mice with nimesulide, nimflumic acid, indomethacin (all PGHS inhibitors, 50 mg/kg i.p. daily) resulted in the inhibition of PGE_2-MØ formation and serum IgE levels. These treatments, in contrast, enhanced IFNγ production in recall responses. Our results indicate that splenic PGE_2-MØ that are induced in Balb/c and C57Bl/6 mice are associated with, or probably induce, a shift from Th1-to-Th2 immune responses 14 days following treatment of mice with 1 mg BCG, corresponding to late stage infection.

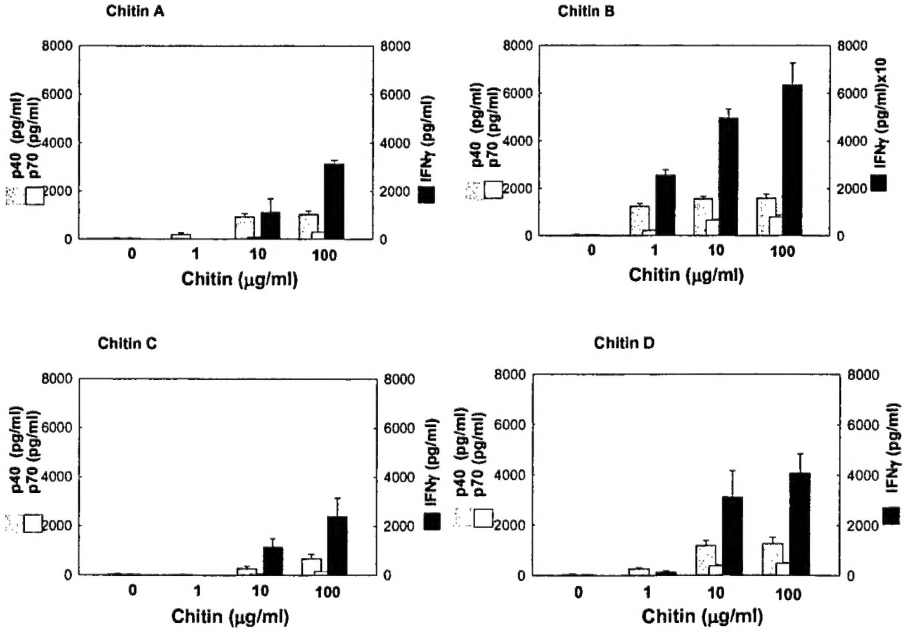
Task 3: To determine if MØ will phagocytose more particles and produce more IL-12 in response to 1 – 4 μm chitin compared to 1 – 10 μm chitin.

a. Determine if MØ treated with 1 – 4 μm chitin particles phagocytose more particles than when treated with an equivalent number of 1 – 10 μm chitin particles (Months 41 – 44).

b. Determine if 1 – 4 μm chitin particles induce more production of IL-12 than an equivalent number of 1 – 10 μm chitin particles (Months 45 – 48).

As described above, we selected a reliable source of Chitin B and prepare 1 – 10 μm chitin particles and 1 – 4 μm particles. Cellular events of phagocytosis that are associated with chitin-induced IL-12 production were characterized (see Figure 1). Abstract "Mechanism of phagocytosed particle-induced IL-12 production in macrophage," will be presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington DC.

Figure 1. 1 – 10 μm chitin particles induced IL-12p40, IL-12p70 (bioactive IL-12) and IFNγ production in spleen cells isolated from IL-10-KO mice. Spleen cells (4 x 10^6 cells in RPMI 1640 + 10% FBS) were incubated with various chitin preparations for 24 hrs. Levels of IL-12p40, IL-12p70 and IFNγ in the culture supernatants were measured by ELISA. Mean ± SD, n=3 Chitin B particles showed higher activities than other chitin particles prepared from Chitin A, Chitin C, and Chitin D.



KEY RESEARCH ACCOMPLISHMENTS:

1. Establishment of chitin particle preparations.
2. Preparations of chitin particles at amounts needed in this project.
3. Characterization of HK-BCG-induced splenic PGE_2-releasing macrophages that eventually down-regulate the Th1 adjuvant activities of HK-BCG.

REPORTABLE OUTCOMES:

Manuscript in preparation.

"Heat-killed BCG enhances host protection against lethal challenges of Listeria monocytogenes," by Y Shibata, P Vos, QN Myrvik. Task 2a (Appendix I)

"High doses of BCG induce splenic PGE2-releasing macrophages and Th1-to-Th2 shifts of immune responses in mice," by Y Shibata, RA Henriksen, I Honda, RM Nakamura, M Smith, QN Myrvik. Task 2a (Appendix II)

"Differential effects of IL-10 on prostaglandin H synthse-2 expression and prostaglandin E2 biosynthesis between spleen and bone marrow macrophages," by Y Shibata, QN Myrvik, RA Henriksen. Task 2a (Appendix III)

Presentations

"High doses of BCG induce splenic PGE_2-releasing macrophages and Th1-to-Th2 shifts of immune responses in mice (I)" by Y Shibata, RA Henriksen, RM Nakamura, I Honda, QN Myrvik, Immunology 2003, 5/6 – 5/10/03, Denver, CO. Task 2a.

"Infections in atherosclerosis --- Mechanisms of Th1-to-Th2 shifts of HSP65 responses," by Y Shibata, the 8th International Symposium on Atherosclerosis, 9/28 – 10/2/03, Kyoto, Japan. Task 2a, (Appendix IV)

"Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE2 production in BCG-immunized mice," by Y Shibata, M Smith, QN Myrvik, H Ohata, A Nishiyama, RA Henriksen, the 2004 Experimental Biology, 4/17 – 4/21/04, Washington DC. Task 2a (Appendix V)

"Mycobacteria-induced osteoclastogenesis and PGE2-releasing macrophage formation in the mouse spleen," by H Ohata, A Nishiyama, RA Henriksen, QN Myrvik, Y Shibata, the 2004 Experimental Biology, 4/17-4/21/04, Washington DC. Task 2a, (Appendix VI)

"Mechanism of phagocytosed particle-induced IL-12 production in macrophage," by A Nishiyama, H Ohata, RA Henriksen, QN Myrvik, Y Shibata, the 2004 Experimental Biology, 4/17-4/21/04, Washington DC. Tasks 1a, 3a and 3b, (Appendix VII)

"Oral administration of N-acetyl-D-glucosamine polymer particles down-regulates allergic responses," by Y Shibata, MR Van Scott, A Nishiyama, H Ohata, QN Myrvik, the CDMRP's Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico. Tasks 1a and 1b, (Appendix VIII).

Employment

PI (Yoshimi Shibata) has had an associate professorship with tenure in the Department of Biomedical Science in the Charles E. Schmidt College of Science at Florida Atlantic University, Boca Raton, Florida, starting September 30, 2003.

CONCLUSIONS:

Mycobacteria and their components have been used as immunotherapeutic agents and Th1 adjuvants due to their exceptional capacities for the induction of cell-mediated immune and Th1 responses. Accordingly, it has been proposed that mycobacteria or their components down-regulate childhood asthma (10-12). However, based on the on-going studies, we believe that the therapeutic effects of chitin particles on endogenous IL-12- or IFNγ- mediated down-regulation of airway allergic responses are greater than those of HK-BCG. Further studies will be conducted in this project.

REFERENCES:

1. Shibata, Y, LA Foster, JF Bradfield, Q N Myrvik. 2000. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. J Immunol 164:1314.

2. Shibata, Y, LA Foster, WJ Metzger, QN Myrvik. 1997. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. Infect Immun 65:1734.

3. Shibata, WJ Metzger, QN Myrvik. 1997. Chitin particle-induced cell mediated immunity is inhibited by mannan --- Mannose receptor-mediated phagocytosis initiates interleukin-12 production. J Immunol 159: 2462.

4. Shibata, Y, I Honda, JP Justice, MR Van Scott, RM Nakamura, QN Myrvik. Shibata Y, Honda I, Justice JP, van Scott MR, Nakamura RM, Myrvik QN. 2001. A Th1-adjuvant --- N-acetyl-D-glucosamine polymer up-regulates Th-1 immunity but down-regulates Th-2 immunity against a mycobacterial protein (MPB-59) in IL-10-knockout and wild type mice. Infect Immun 69:6123-6130.

5. Shibata Y, Volkman A. 1985. The effect of bone marrow depletion on prostaglandin E-producing suppressor macrophages in mouse spleen. J Immunol. 135:3897.

6. Druker BJ, Wepsic HT. 1983. BCG-induced macrophages as suppressor cells. Cancer Invest 1:151.

7. Baek KH, Ha SJ, Sung YC. 2001. A novel function of phosphorothioate oligodeoxynucleotides as chemoattractants for primary macrophages. J Immunol.167:2847-54.

8. Chen Y, Zhang J, Moore SA, Ballas ZK, Portanova JP, Krieg AM, Berg DJ. 2001. CpG DNA induces cyclooxygenase-2 expression and prostaglandin production. Int Immunol. 13:1013-20.

9. Sparwasser T, Hultner L, Koch ES, Luz A, Lipford GB, Wagner H. 1999. Immunostimulatory CpG-oligodeoxynucleotides cause extramedullary murine hemopoiesis. J Immunol. 162:2368-74.

10. Shirakawa, T, T Enomoto, S Shimazu, JM Hopkin. 1997. The inverse association between tuberculosis responses and atopic disorder. Science 275:77.

11. Sano K, Haneda K, Tamura G, Shirato K. 1999. Ovalbumin (OVA) and Mycobacterium tuberculosis bacilli cooperatively polarize anti-OVA T-helper (Th) cells toward a Th1-dominant phenotype and ameliorate murine tracheal eosinophilia. Am J Respir Cell Mol Biol 20:1260.

12. Nahori MA, Lagranderie M, Lefort J, Thouron F, Joseph D, Winter N, Gicquel B, Lapa E, Silva JR, Vargaftig BB. 2001. Effects of Mycobacterium bovis BCG on the development of allergic inflammation and bronchial hyperresponsiveness in hyper-IgE BP2 mice vaccinated as newborns. Vaccine 19:1484.

APPENDICES:

Appendix I: Y Shibata, P Vos, QN Myrvik, Heat-killed BCG enhances host protection against lethal challenges of Listeria monocytogenes, manuscript in preparation.

Appendix II: Y Shibata, RA Henriksen, I Honda, RM Nakamura, M Smith, QN Myrvik, High doses of BCG induce splenic PGE2-releasing macrophages and Th1-to-Th2 shifts of immune responses in mice, manuscript in preparation.

Appendix III: Y Shibata, QN Myrvik, RA Henriksen , Differential effects of IL-10 on prostaglandin H synthse-2 expression and prostaglandin E2 biosynthesis between spleen and bone marrow macrophages, manuscript in preparation.

Appendix IV: Y Shibata, M Smith, JF Bradfield, Infections in atherosclerosis --- Mechanisms of Th1-to-Th2 shifts of HSP65 responses, Abstract, the 8th International Symposium on Atherosclerosis, 9/28 – 10/2/03, Kyoto, Japan.

Appendix V: Y Shibata, M Smith, QN Myrvik, H Ohata, A Nishiyama, RA Henriksen, Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE_2 production in BCG-immunized mice," Abstract was accepted and will be presented at the 2004 Experimental Biology, 4/17 – 4/21/04, Washington DC.

Appendix VI: H Ohata, A Nishiyama, RA Henriksen, QN Myrvik, Y Shibata, Mycobacteria-induced osteoclastogenesis and PGE2-releasing macrophage formation in the mouse spleen, Abstract was accepted and will be presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington DC.

Appendix VII: A Nishiyama, H Ohata, RA Henriksen, QN Myrvik, Y Shibata. Differentiation of signaling pathways involved in chitin- and LPS-induced IL-12 production by macrophages. Abstract was accepted and will be presented at the 2004 Experimental Biology Meeting, 4/17-21, 2004, Washington, DC.

Appendix VIII: Y Shibata, MR Van Scott, A Nishiyama, H Ohata, QN Myrvik, Oral administration of N-acetyl-D-glucosamine polymer particles down-regulates allergic responses, Abstract was accepted and will presented at the CDMRP's Military Health Research Forum (MHRF), 4/25-4/28/04, 2004, San Juan, Puerto Rico.

Heat-killed BCG enhances host protection against lethal challenges of *Listeria monocytogenes*

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Running title: Anti-listeriosis by heat-killed BCG

ABSTRACT

Resistance to infection with *Listeria monocytogenes* involves multiple interactions including activated macrophages (MØ) and neutrophils, many of which can be modulated by *Mycobacterium bovis* BCG. Previous studies show that a high dose (1 mg) heat-killed (HK) BCG develops a switch from Th1 to Th2 immune responses that potentially down-regulates listericidal MØ activities. On the other hand, HK-BCG induces neutrophil production and activation that are listericidal. In this study, we examined the effects of HK-BCG on host defense mechanisms against *L. monocytogenes* challenges. Balb/c mice were given 0.01 mg or 1 mg HK-BCG ip and challenged ip with lethal doses (10^6, 10^7 or 10^8 CFU) of *L. monocytogenes* 14 days after HK-BCG immunization. Our results showed that HK-BCG enhanced host protection in a dose-dependent manner. HK-BCG at 1 mg dose protected hosts completely from over 100 LD_{50} (10^8 CFU) challenge of *L. monocytogenes*. The protective effect of BCG was decreased significantly by anti-neutrophil antibody (RB6-8C5). Our results demonstrate that a high dose of HK-BCG establishes neutrophil-mediated host defense mechanisms that protect against lethal doses of *L. monocytogenes*, despite the HK-BCG induced Th2 immune response that appears to suppress bactericidal MØ activation. The long-term belief that MØ activation in cell-mediated immune responses is essential for innate immune protection is contradicted by our findings for listeriosis.

INTRODUCTION

Listeria monocytogenes is a Gram-positive, facultative, intracellular pathogen capable of causing severe infections characterized by high mortality in immunocompromised humans including pregnant women, new-born infants and children (1,2,3). Listeriosis in mice is a model used widely to explore factors that regulate host defenses against intracellular pathogens including mycobacteria (4,5). Listericidal macrophages (MØ) that develop following an adoptively transferable cell-mediated immune (CMI) response are thought to be major effector cells against *Listeria monocytogenes* (5). However, the initial stage of protection (innate immunity) against listeriosis involves neutrophils (6). Although new strategies and anti-listeriosis pharmaceuticals are needed to prevent this infection, the effects of immunomodulatory agents on listeriosis remain unclear and controversial.

Live *Mycobacterium bovis* BCG induces tumoricidal activity in the host (7) and has been widely used for treatment of superficial bladder cancer (7, 8). Live BCG also induces a non-specific microbicidal MØ activity against *L. monocytogenes* (9,10,11,12) and *Toxoplasma gondii* (10). However, Wing (13) showed that high doses of live BCG, which suppresses CMI, decrease resistance to *L. monocytogenes* infection in mice. The differential effects of BCG treatments among the studies would be due to various levels of host immune responses induced by BCG.

Hosts develop Th1 immunity at the early stages of infection against mycobacteria such as *M. tuberculosis* and *M. leprae*. In some instances, however, a switch to Th2 immunity is observed

during progressive stages of infections (14,15,16,17). BCG induces myelopoiesis (18,19) that potentially protects hosts against listeriosis (12,20). The cooperation between neutrophils and MØ in the killing of *L. monocytegenes* appears to be important (12). Since live BCG treatment, inducing significant Th1 responses, is more protective than killed BCG (11), neutrophil-mediated protection would still require a Th1-mediated microbicidal MØ activation.

We established a model of Th1-to-Th2 shifts of immune responses in mice given selected doses heat-killed (HK)-BCG, since mycobacterial cell wall components appear to be involved in the mechanisms of the shifts (21). A low dose (0.01 mg/dose) of HK-BCG induces Th1-mediated MØ activation, whereas a high dose (1 mg/dose) HK-BCG inhibits Th1 responses while enhancing Th2-mediated antibody formation (22). High dose HK-BCG induces splenic PGE_2-releasing MØ that, at least in part, regulate the Th1-to-Th2 shifts of immune responses (21). Activated Th2 cells produce IL-4 and IL-10, both of which inhibit Th1-mediated microbicidal MØ activation (23,24). In addition, PGE_2 inhibits bactericidal and tumoricidal MØ functions (18). It would be reasonable to speculate that HK-BCG impairs Th1-mediated microbicidal MØ activation and, therefore, down-regulates host defense mechanisms against *L. monocytogenes* (3). In this report, we examine the effects of HK-BCG on host defense against lethal challenges of *L. monocytogenes*. Our results show that HK-BCG enhances host protection that appears to be associated with increases in neutrophils but is independent of the shifts from Th1 to Th2 response against BCG.

MATERIALS AND METHODS

Mice. Non-pregnant female Balb/c mice, 8 weeks old, were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water *ad libitum*. IACUC of East Carolina University Brody School of Medicine approved experimental protocols employed in this study.

Preparations of HK-BCG. The cultured bacteria of *M. bovis* Calmette-Guerin bacillus (BCG) Tokyo 172 strain were washed, autoclaved, and lyophilized as described previously (26). The powder of HK-BCG was suspended in saline immediately before use. The suspensions of HK-BCG were dispersed by brief sonication (10s) prior to injection. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma) (22).

***Listeria monocytogenes* and LD_{50} determination.** *Listeria monocytogenes* 10403S (25) was obtained from Dr. Julie Theriot, Stanford University. The bacteria were grown in brain-heart infusion broth (Difco) at 37°C. The bacteria were harvested in the logarithmic phase of growth and washed 3 times with cold saline and re-suspended in saline at 2×10^9 CFU/ml. Aliquot was stored at -80°C. CFU per milliliter were determined by plating dilutions of the cultures on brain heart infusion agar plates. For LD_{50} determinations, groups of 5 mice were treated ip with 10-fold dilutions (in saline) of the bacterial suspensions following thawing. Mice were observed for 12 days, after which time no more deaths occurred. LD_{50} values were calculated by the method of

Reed and Muench (26).

Treatment of mice. Groups of mice received ip doses (0.01 or 1 mg/dose) of HK-BCG (5 mice/group) on day 0. Controls received 0.2 ml of saline. On day 14, mice were challenged ip with *Listeria monocytogenes* at 10^6, 10^7, and 10^8 CFU. Mortality was recorded twice daily until day 26. On day 26, mice were euthanized and spleens, livers and lungs were isolated from mice. Organs were homogenized in 1 ml of PGS, and serial dilutions of the homogenates were plated on agar plates. Colonies were counted after incubation at 37°C for 24 h.

Antibody and depletion procedure. The neutrophil-depleting monoclonal antibody RB6-8C5 was a gift from Dr. Robert Coffman (27). The hybridoma RB6-8C5 was grown in culture and monoclonal antibody affinity-purified over a Protein G-column (Pharmacia, Uppsala, Sweden) before use. For *in vivo* neutrophil deletion, mice were treated ip with 100 μg of monoclonal antibody RB6-8C5 1 day before *Listeria* infection. Depletion was verified cytometrically described below.

Cytometric detection. Spleens from each group of mice were isolated and pooled. Excised spleens were minced with scissors and digested with collagenase D (C2139, Sigma) at 50 U/ml in RPMI 1640 plus 10% FBS at 37° C for 60 min and filtered through a 100-μm mesh. Single cell suspensions were prepared by washing digested cells with RPMI 1640 containing DNase at 100 μg/ml (DN-25, Sigma). Spleen cells were characterized by flow cytometry following staining with monoclonal antibodies prepared in rat against RB6-8C5 (neutrophil), Mac-1 (β2 integrin),

CD16/32 (Fcγ receptor), ER-TR9 (marginal zone MØ), B220 (B cell) and TLR4 (toll-like receptor 4). The presence of the primary antibody was determined by phycoerythrin (PE) -conjugated donkey anti-rat IgG/IgM at 1:250 dilution (Jackson ImmunoResearch, West Grove, PA). Single nucleated cells were defined by forward and sideward scatter pattern and gated to exclude debris from analysis.

Total IgE and HSP65-specific antibodies in sera. Total serum IgE levels were detected by ELISA using purified mouse IgE κ isotype (PharMingen, San Diego, CA) as a standard and rat anti-mouse IgE monoclonal antibody, clone R35-72 (PharMingen) as a capture antibody. Purified protein derivatives (PPD)- and mycobacterial heat-shock protein 65 (HSP65)-specific IgG1 and IgG2a levels were measured by ELISA. Plates were coated with 0.5 μg/ml PPD (Japan BCG Lab, Tokyo) or 0.1 μg/well HSP65 (StressGen, BC, Canada) in 0.05 M sodium carbonate buffer, pH 9.6, overnight at 4°C. Biotinylated rat monoclonal antibodies detecting IgG1 and IgG2a were clones A85-1 and R19-15, respectively (PharMingen) (22).

Statistics. Data are expressed as mean ± standard deviation. Differences between groups for serum antibody levels were analyzed by one-way analysis of variance. The log rank test was used to determine the significance of difference in survival rate. A *p*-value of less than 0.05 is considered statistically significant.

RESULTS

HK-BCG-immunized mice were protected from lethal challenges of *L. monocytogenes*. To determine whether HK-BCG treatment altered protective responses against *L. monocytogenes* infection, Balb/c mice were immunized ip with 0.01 mg or 1 mg/dose HK-BCG and challenged with *L. monocytogenes* at either 10^6, 10^7, or 10^8 CFU. Mice treated with saline served as controls. All control mice succumbed to these high *Listeria* challenge inoculums. As shown in Figure 1, 1 mg HK-BCG completely protected mice from 10^8 CFU challenge, a 100 LD_{50} dose. HK-BCG at 0.01 mg/dose showed a significant protection from 10^7 CFU, but not 10^8 CFU challenge. All live mice that were immunized with HK-BCG and challenged with *Listeria* showed undetectable CFU levels (<10 CFU) in the spleen, lung and liver 12 days after the challenges of *Listeria* (data not shown). The results clearly demonstrated that Balb/c mice immunized with 1 mg HK-BCG were protected from all lethal *L. monocytogenes* challenges.

Expression of Th2 phenotype in the high dose HK-BCG-treated Balb/c mice. Previous studies (28,29) indicated that mycobacterial products increase PGE_2 release from splenic macrophages (PGE_2-M∅) in mice. In the spleen, PGE_2 inhibits Th1 responses against mycobacterial antigens with enhanced Th2 responses in the spleen (21,30). Endogenous IL-4, a Th2 cytokine, and IFNγ, a Th1 cytokine, are isotype-switching signals for antigen-specific B cells, which increase serum IgE and IgG1 and serum IgG2a, respectively (31,32). Therefore, we determined if these heavy-chain class switches were induced by HK-BCG immunization. As shown in Table 1, Balb/c mice that were treated with 1 mg HK-BCG expressed serum IgG1

against mycobacterial antigens (PPD and HSP65) and increased total serum IgE. In contrast, 0.01 mg HK-BCG only induced IgG2a against HSP65.

HK-BCG induced splenic neutrophil production at a dose-dependent manner. Mycobacterial components induce myelopoiesis (18). To enumerate neutrophil production detected cytometrically by RB6-8C5 antibody, spleen cells were prepared from Balb/c mice that were treated with HK-BCG at 0 (saline), 0.01 and 1 mg/dose. The high dose produced a 5-fold increase in spleen weight after 14 days. However, the low dose did not produce any detectable levels of splenomegaly. As shown in Table 2, RB6-8C5$^+$ cells were increased by 40-fold following 1 mg HK-BCG and 2.5-fold following 0.01 mg treatment. Mac-1$^+$, CD16/32$^+$, ER-TR9$^+$, TLR4$^+$, or B220$^+$ cells were also increased significantly in the high dose HK-BCG group.

Treatment with the anti-neutrophil monoclonal antibody RB6-8C5 impaired the resistance of HK-BCG-immunized mice. Previous reports (6,33,34) have established that the treatment with RBC-8C5 antibody reduces neutrophil-mediated resistance to listeriosis. To assess whether the increases in protection against lethal doses of *L. monocytogenes* are dependent on HK-BCG-induced neutrophils, HK-BCG-immunized mice were further treated with RB6-8C5 antibody one day before *L. monocytogenes* challenge. As shown in Figure 2, the treatment with RB6-8C5 resulted in a loss of the protective effect of HK-BCG. The treatment significantly eliminated RB6-8C5$^+$ cells and Mac-1$^+$ cells but not CD16/32$^+$, or TLR4$^+$ cells (Table 3).

DISCUSSION

Previous studies (11,35) suggested that treatment of mice with HK-BCG or related products induces no significant protection against listeriosis, because HK-BCG induces "suppressor" mechanisms down-regulating listericidal MØ activation. In addition, compared to live BCG, HK-BCG does not induce sufficient iNOS and IFNγ production to inhibit the proliferation of *L. monocytogenes* (11). It was not predicted, therefore, that a 1 mg dose of HK-BCG, bacterial populations comparable to those in the late stage infection, would induce host resistance against a lethal challenge (>100 LD_{50}) of *L. monocytogenes*. These provocative findings presented that HK-BCG-induced protective mechanisms appear to be mediated by increases in neutrophils. Our HK-BCG study provides quantitative analyses of the mechanisms underlying live BCG-mediated protection against listeriosis (9,10,11) are at least in part due to increases in RB6-8C5$^+$ neutrophils (12).

Heat-resistant cell wall mycoloyl glycolipids in BCG or *Corynebacterium parvum* (*Propionibacterium acnes*) are known to induce "suppressor cells" including PGE_2-MØ (22,29,35,36). Splenic PGE_2-MØ induce a shift of Th1-to-Th2 responses (21). An interesting finding was that the 0.01 mg dose of HK-BCG induced Th1 lymphocyte responses against mycobacteria, although the effects are not enough to protect against tuberculosis (37). In this study, we found that a 1 mg dose of HK-BCG induced not only an increased total serum IgE level but also serum IgG1 and IgG2a antibody formation against mycobacteria, indicating a mixed Th1 and Th2 immune response. Th2 immune responses result in reduced bactericidal MØ activities

against intracellular bacteria (23,24). Our results clearly demonstrate, however, that high dose HK-BCG-induced Th2 responses do not down-regulate host anti-listeriosis.

Listeriosis is ubiquitous in nature, and only infects people whose anti-microbial defenses have been in some way compromised. Host protective mechanisms against listeriosis is considered to be similar to those against tuberculosis (4,5), although tuberculosis and listeriosis are initially acquired as respiratory and G.I. tract pathogens, respectively. It is significant, however, that, unlike tuberculosis, listeriosis is not a common infection in patients with AIDS. This would indicate that anti-*Listeria* resistance involves, at least in part, more than up-regulation of MØ activation by Th1 cytokines secreted by *Listeria*-specific CD4 lymphocytes as was believed to be the basis of immunity in mice (38).

HK-BCG-induced neutrophils likely migrate to foci of listeriosis, where they exert microbicidal function. Although neutrophils can release large amounts of IL-12 leading to Th1-mediated listericidal immunity (39), our results do not support this possibility. Gregory et al (20) showed that, in systemic listeriosis, liver neutrophils phagocytose *L. monocytogenes* and undergo apoptosis, followed by phagocytosis and removal by neighboring Kupffer cells through the interaction mediated by Mac-1 and ICAM-1 mechanisms. It remains to be elucidated whether similar anti-listeriosis mechanisms are significantly enhanced not only in the liver but also other tissues when immunized with BCG (12).

In conclusion, our findings demonstrate that despite the induction of a Th2 immune response,

HK-BCG-induced neutrophils mediate the host defense mechanism against a lethal challenge of *L. monocytogenes*. It is clear that more information is needed concerning the HK-BCG-induced protection against listeriosis. However, our studies provide new insights into the importance of investigating HK-BCG-induced immunomodulations that might have importance in the immunocompromised populations.

Acknowledgement.

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REFERENCES

1. Jones, D. (1990) Foodborne listeriosis. Lancet 336,1171-1174.
2. Schuchat, A., Deaver, K.A., Wenger, J.D., Plikaytis, B.D., Mascola, L., Pinner, R.W. Reingold, L., Broome, C.V. (1992) Role of foods in sporadic listeriosis. I. Case-control study of dietary factors. JAMA 267, 2041-2045.
3. Rocourt, J., Jacquet, C., Reilly, A. (2000) Epidemiology of human listeriosis and seafoods. Inter. J. Food Microbiol. 62,197-209.
4. Kaufmann, S.H.E. (1993) Immunity to intracellular bacteria. Annu. Rev. Immunol. 11,129-163.
5. Mackaness, G.B. (1962) Cellular resistance to infection. J. Exp. Med. 116, 381-406.
6. Rogers, H.W., Unanue, E.R. (1993) Neutrophils are involved in acute, nonspecific resistance to *Listeria monocytogenes* in mice. Infect. Immun. 61, 5090-5096.
7. Morales, A., Eidinger, D., Bruce, A.W. (1976) Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. J. Urol. 116,180-183.
8. Kuroda, K., Brown, E.J., Telle, W.B., Russel, D.G., Ratliff, T.L. (1993) Characterization of the internalization of Bacillus Calmette-Guerin by human bladder tumor cells. J. Clin. Invest. 91, 69-76.
9. Simon, H.B., Sheagren, J.N. (1971) Cellular immunity in vitro. I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. 133, 1377-1389.
10. van Dissel, J.T., Stikkelbroek, J.J., van den Barselaar, M.T., Sluiter, W., Leijh, P.C., van Furth, R. (1987) Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages. J. Immunol. 139,1665-1672.

11. Yang, J., Kawamura, I., Mitsuyama, M. (1997) Involvement of inflammatory cytokines and nitric oxide in the expression of non-specific resistance to *Listeria monocytogenes* in mice induced by viable but not killed *Mycobacterium bovis* BCG. Microb. Pathog. 22, 79-88.

12. Leal, I.S., Appelberg, R., Silva, M.T. (1996) Neutrophils are involved in the non-specific resistance to listeriosis induced by mycobacterial infections. Immunology 89, 442-448.

13. Wing, E.J. (1981) Bacillus Calmette-Guerin (BCG) decreases resistance to *Listeria monocytogenes* infection in mice. Immunology 44, 509-515.

14. Huygen, K., Abramowicz, D., Vandenbussche, P. Jacobs, F., De Bruyn, J., Kentos, A., Drowart, A., Van Vooren J.P., Goldman, M. (1992) Spleen cell cytokine secretion in *Mycobacterium bovis* BCG-infected mice. Infect. Immun. 60, 2880-2886.

15. Surcel, H.M., Troye-Blomberg, M., Paulie, S., Andersson, G., Moreno, C., Pasvol, G., Ivanyi, J. (1994) Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. Immunology 81,171-176.

16. Yamamura, M., Uyemura, K., Deans, R.J., Weinberg, K., Rea, T.H., Bloom, B.R., Modlin, R.L. (1991) Defining protective responses to pathogens: cytokine profiles in leprosy lesions. Science 254, 277-279.

17. Yong, A.J., Grange, J.M., Tee, R.D., Beck, J.S., Bothamley, G.H., Kemeny, D.M., Kardjito, T. (1989) Total and anti-mycobacterial IgE levels in serum from patients with tuberculosis and leprosy. Tubercle 70, 273-279.

18. Matthys, P., Vermeire, K., Heremans, H., Billiau, A. (2000) The protective effect of IFN-gamma in experimental autoimmune diseases: a central role of mycobacterial adjuvant-induced myelopoiesis. J. Leukoc. Biol. 68, 447-454.

19. Urbaschek, R., Mannel, D.N., Urbaschek, B. (1987) Tumor necrosis factor induced stimulation of granulopoiesis and radioprotection. Lymphokine Res. 6, 179-186.

20. Gregory, S.H., Cousens, .P., van Rooijen, N., Dopp, E.A., Carlos, T.M., Wing, E.J. (2002) Complementary adhesion molecules promote neutrophil-Kupffer cell interaction and the elimination of bacteria taken up by the liver. J. Immunol. 168, 308-315.

21. Shibata, Y., Henriksen, R.A., Nakamura, R.M., Honda, I., Myrvik, Q.N. (2003) High doses of BCG induce splenic PGE2-releacing macrophages and Th1-toTh2 shifts of immune responses in mice. FASEB J. 17,C17.

22. Shibata, Y., Honda, I., Justice, J.P., van Scott, M.R., Nakamura, R.M., Myrvik, Q.N. (2001) A Th1-adjuvant --- N-acetyl-D-glucosamine polymer up-regulates TH-1 immunity but down-regulates Th-2 immunity against a mycobacterial protein (MPB-59) in IL-10-knockout and wild type mice. Infect. Immun. 69, 6123-6130.

23. Hsieh, C.S., Heimberger, A.B., Gold, J.S., O'Garra, A., Murphy, K.M. (1992) Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. Proc. Natl. Acad. Sci. USA 89, 6065-6069.

24. Oswald, I.P., Gazzinelli, R.T.. Sher, A., James, S.L. (1992) IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. J. Immunol. 148, 3578-3582.

25. Portnoy, D.A., Jacks, P.S., Hinrichs, D.J. (1988) Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. J. Exp. Med. 167, 1459-1471.

26. Reed, L.J., Muench, H. (1938) A simple method of estimating 50 per cent endpoints. Am. J. Hyg. 27, 493-497.

27. Tepper, I., Coffman, T.L., Leder, P. (1992) An eosinophil-dependent mechanism for the antitumor effect of interleukin-4. Science 257, 548-551.

28. Druker, B.J., Wepsic, H.T. (1983) BCG-induced macrophages as suppressor cells. Cancer Invest. 1, 151-161.

29. Shibata, Y., Volkman, A. (1985) The effect of bone marrow depletion of prostaglandin E producing suppressor macrophages in mouse spleen. J. Immunol. 135, 3897-3904.

30. Chen, C.H., Zhang, D.H., LaPorte, J.M., Ray, A. (2000) Cyclic AMP activates p38 mitogen-activated protein kinase in Th2 cells: phosphorylation of GATA-3 and stimulation of Th2 cytokine gene expression. J. Immunol. 165, 5597-5605.

31. Coffman, R.L., Ohara, J., Bond, M.W., Carty, J., Zlotnik, A., Paul, W.E. (1986) B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. J. Immunol. 136, 4538-4541.

32. Snapper, C.M., Paul, W.E. (1987) Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 236, 944-947.

33. Conlan, J.W., North, R.J. (1994) Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody. J. Exp. Med. 179, 259-268.

34. Gregory, S.H., Sagnimeni, A.J., Wing, E.J. (1996) Bacteria in the bloodstream are trapped in the liver and killed by immigrating neutrophils. J. Immunol. 157, 2514-2520.

35. Wing, E.J., Kresefsky-Friedman, D.Y. (1980) Decreased resistance to *Listeria monocytogenes* in mice injected with killed *Corynebacterium parvum*: association with suppression of cell-mediated immunity. J. Infect. Dis. 141, 203-211.

36. Han, Y., Kawada, N., Yano, I. (1998) Granuloma formation and in vitro macrophage activation in mice by mycoloyl glycolipids from *Nocardia asterides* and related taxa. Osaka City Med. J. 44, 201-217.

37. McDonough, K.A., Kress, Y., Bloom, B.R. (1993) Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect. Immun. 61, 2763-2773.

38. North, R.J., Dunn, P.L., Conlan, J.W. (1997) Murine listeriosis as a model of antimicrobial defense. Immunol. Rev. 158, 27-36.

39. Bliss, S.K., Butcher, B.A., Denkers, E.Y. (2000) Rapid recruitment of neutrophils containing prestored IL-12 during microbial infection. J. Immunol. 165, 4515-4521.

Table 1. HK-BCG induced serum IgG1 specific against mycobacterial antigens and total IgE[a].

	0 (saline)	0.01 mg BCG	1 mg BCG
Total serum IgE (ng/ml)	810 ± 270	804 ± 105	2,070 ± 900**
PPD-specific IgG1 titer ($A_{450\ nm}$)	0.07 ± 0.01	0.08 ± 0.01	0.79 ± 0.32*
PPD-specific IgG2a titer ($A_{450\ nm}$)	0.07± 0.01	0.07 ± 0.01	0.26 ± 0.06*
HSP65-specific IgG1 titer ($A_{450\ nm}$)	0.02 ± 0	0.06 ± 0.02*	1.35 ± 0.03**
HSP65-specific IgG2a titer ($A_{450\ nm}$)	0.01 ± 0.01	0.46 ± 0.17*	1.33 ± 0.36**

[a]Balb/c female mice (8 week-old) were given ip HK-BCG at 0 (0.2 ml of saline), 0.01 mg, or 1 mg. Fourteen days after the treatments, sera were harvested. Total serum IgE levels were measured as described in the *Materials and Methods* section. Following dilutions at 1:5 and 1:100 of sera, IgG2a levels against PPD or HSP65 and IgG1 against PPD or HSP65, respectively, were measured. Mean ± SD, n=5 Each result represents a group of 5 mice from three experiments. * and **, $p<0.05$ and $p<0.01$ compared to the saline group.

Table 2. RB6-8C5$^+$ spleen cells in BCG-immunized mice[a].

	Cell number x 10^7 (%)[b]		
	0 (saline)	0.01 mg BCG	1 mg BCG
Total nucleated cells	85 (100)	92 (100)[b]	426 (100)
RB6-8C5$^+$ cells	4 (5)	10 (11)	166 (39)
Mac-1$^+$ cells	14 (16)	17 (18)	200 (47)
CD16/32$^+$ cells	3 (4)	6 (6)	107 (25)
ER-TR9$^+$ cells	3 (4)	4 (4)	26 (6)
B220$^+$ cells	55 (65)	68 (74)	337 (79)
TLR4$^+$ cells	10 (12)	17 (18)	251 (59)

[a]Balb/c female mice (8 week-old) were given ip HK-BCG at 0 (0.2 ml of saline), 0.01 mg, or 1 mg. Fourteen days after the treatments, spleens were harvested and digested with collagenase D (50 U/ml, 60 min, 37°C). Nucleated spleen cells were stained with PE-conjugated antibodies against MØ surface antigens indicated. Fluorescence of 10^4 stained cells were measured using a FACScan flow cytometer and the FACScan research program (Becton-Dickinson). Results are from 4 experiments.

[b]% positive cells are indicated in the parentheses.

Table 3. The effects of anti-neutrophil antibody on spleen cell populations[a].

	% Suppression
Total nucleated cells	NS[b]
RB6-8C5$^+$ cells	73
Mac-1$^+$ cells	62
CD16/32$^+$ cells	NS
ER-TR9$^+$ cells	8
B220$^+$ cells	NS
TLR4$^+$ cells	NS

[a]Balb/c female mice (8 week-old) were given ip HK-BCG at 1 mg day 0. Mice were further treated with anti-RB6-8C5 antibody or rat IgG as controls at 100 μg ip day 14. Day 15, spleen cells were isolated and stained with antibodies as described in Table 2. The results were compared between anti-RB6-8C5 treatment group and rat IgG controls and expressed as % suppression.

[b]NS: no suppression.

Legends for Figures

Figure 1. Effects of HK-BCG treatments on resistance to *L. monocytogenes* infection at lethal and sub-lethal doses. Mice were injected ip on day 0 with HK-BCG at 0 (saline) (▫), 0.01 mg (□) or1 mg/dose (•) day 0. They were infected with 10^6 CFU, 10^7 CFU and 10^8 CFU of *L. monocytogenes* on day 14. Mortality was recorded twice daily. Results are for 5 mice in each of two separate experiments. The differences of survival data between each HK-BCG group and saline control group were statistically significant ($p<0.001$; log rank test).

Figure 2. Effects of monoclonal antibody RB6-8C5 administration on survival of mice after ip inoculation with *L. monocytogenes*. Five mice in each group received 100 μg of monoclonal antibody RB6-8C5 (●) or rat IgG (O) ip 24 h before ip inoculation with 10^8 CFU *L. monocytogenes*. Mice were then monitored for death twice daily. The differences of survival data between the treatments with RBC-8C5 antibody and rat IgG were statistically significant ($p<0.05$; log rank test).

Figure 1.

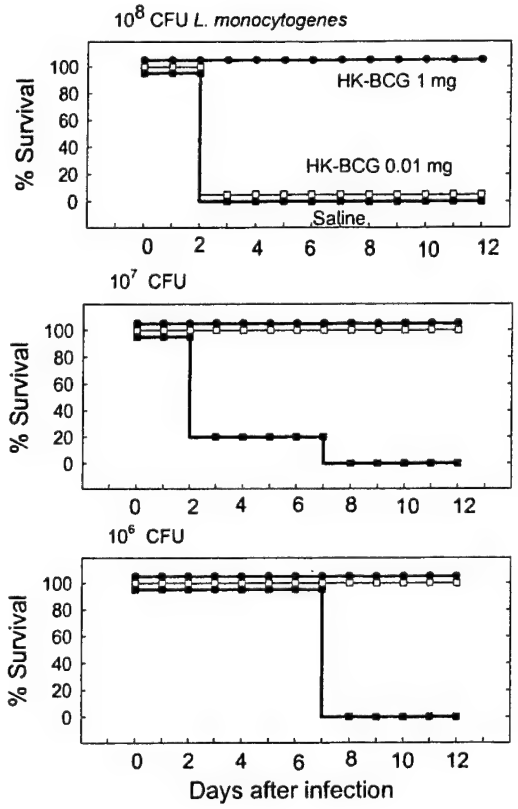
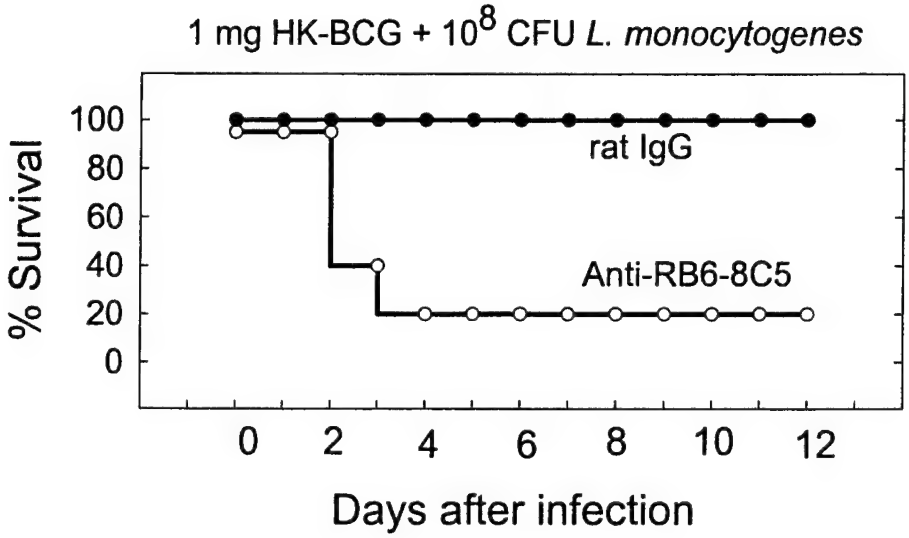

10^8 CFU *L. monocytogenes*
% Survival
100
80
60
40
20
0
HK-BCG 1 mg
HK-BCG 0.01 mg
Saline
0 2 4 6 8 10 12
10^7 CFU
% Survival
100
80
60
40
20
0
0 2 4 6 8 10 12
10^6 CFU
% Survival
100
80
60
40
20
0
0 2 4 6 8 10 12
Days after infection

Figure 2.


1 mg HK-BCG + 10^8 CFU L. monocytogenes
% Survival
100
80
60
40
20
0
rat IgG
Anti-RB6-8C5
0
2
4
6
8
10
12
Days after infection

High doses of BCG induce splenic PGE2-releasing macrophages and Th1-to-Th2 shifts of immune responses in mice

Running title: PGE_2-MØ and Th1-to-Th2 shifts

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ABSTRACT

Hosts develop Th1 immunity during the early stages of mycobacterial infections, but induce a switch to Th2 immunity during the later stage of infection. Mycobacterial products induce PGE_2-releasing macrophages (PGE_2-MØ) in the mouse spleen. Since PGE_2 inhibits Th1 responses with enhanced Th2 responses, we determined whether splenic PGE_2-MØ induce this immune shift. Heat-killed *Mycobacterium bovis* BCG at 0.01 and 1 mg/dose were given i.p. to Balb/c and C57B1/6 mice. Splenic PGE_2-MØ were characterized by the *ex vivo* release of PGE_2 (>1 ng/10^6 cells) and the expression of prostaglandin G/H synthase (PGHS) -1, PGHS-2, membrane PGE synthase (PGES) and cytosolic PGES. PGE_2-MØ were detected 14 days after treatment with 1 mg, but not with 0.01 mg, BCG. BCG-treated mice also expressed (i) serum IgG1 and IgE antibodies against mycobacterial antigens and (ii) IL-4 and IL-10 production by spleen cells stimulated *ex vivo* with mycobacterial antigens (recall responses). Treatment of BCG-injected mice with nimesulide, nimflumic acid, indomethacin (all PGHS inhibitors, 50 mg/kg i.p. daily) resulted in the inhibition of PGE_2-MØ formation and serum IgE levels. These treatments, in contrast, enhanced IFNγ production in recall responses. Our results indicate that splenic PGE_2-MØ that are induced in Balb/c and C57Bl/6 mice are associated with, or probably induce, a shift from Th1-to-Th2 immune responses 14 days following treatment of mice with 1 mg BCG, corresponding to late stage infection.

INTRODUCTION

Hosts develop Th1 cellular immunity in the early stages of infection by mycobacteria such as *Mycobacterium tuberculosis* and *M. leprae*, but in some instances, a switch to Th2 immunity is observed during the later progressive stages of infections (1-3). Mycobacterial components have been employed as adjuvants (for example Freund's complete adjuvant with *M. tuberculosis*) to induce autoimmune diseases such as adjuvant arthritis (4) and other chronic inflammatory diseases (5). The adjuvants enhance Th1-mediated MØ activation and Th2-mediated antibody formation (6). The mechanisms of the Th1-to-Th2 shift of lymphocyte responses is not well understood. *M. bovis* bacillus Calmette-Guerin (BCG), an attenuated vaccine for tuberculosis, is a classic immunomodulator used for a cancer immunotherapy (7), and induces a Th1-to-Th2 shift of immune responses in a dose dependent manner (8). Therefore, animals treated with BCG provide a unique opportunity to study the mechanism of such immunological shifts.

Activated Th2 cells produce IL-4 and IL-10, both of which inhibit Th1-mediated microbicidal MØ activation (9,10). Th2 cells mediate IgG1 and IgE antibody formation against mycobacterial antigens. IgE immune complexes activate mast cells and eosinophils via high affinity Fc epsilon receptors to cause tissue damages (11). In addition, Th1 cell-mediated host responses against mycobacterial heat-shock protein 65 (HSP65) are protective against *M. tuberculosis* infection (12). However, Th1-to-Th2 shifts of immune responses against mycobacteria result in the formation of antibodies against HSP65 (13). Anti-HSP65 plays a non-protective role and may contribute to atherosclerosis by attacking arteries expressing endogenous HSP60 autoantigen through antigenic mimicry (14). Therefore, Th1-to-Th2 shifts of immune responses may be

associated with the pathogenesis of various chronic diseases, although the consequences of this Th1-to-Th2 shift in controlling mycobacterial infections are still unclear (15-17).

The spleen is the secondary infection site of mycobacteria and plays a systemic regulatory role in developing acquired immune responses against these pathogens. Unlike other tissue MØ isolated from peritoneum, bone marrow or blood, normal splenic MØ exhibit relatively low levels of PGE_2-releasing capacity (<1 ng of $PGE_2/10^6$ MØ) (18,19). However, splenic PGE_2-releasing MØ (PGE_2-MØ) are increased by mycobacterial infections and other chronic disease conditions in which Th1-to-Th2 shifts are observed (20-24). Previous studies (6,18,19) indicate that heat-killed (HK) BCG and *Corynebacterium parvum (Propionibacterium acnes)*, in a dose dependent manner, induce splenic PGE_2-MØ (>5 ng/10^6 MØ) within 5 – 21 days. Furthermore, the formation of splenic PGE_2-MØ is dependent on radiosensitive bone marrow cells but independent of circulating monocytes (18,19). However, further characterization of MØ phenotypes and the requirements for PGE_2 synthesis are needed.

It is well established that PGE_2 strongly inhibits the production of Th1 cytokines such as IL-2, IL-12 and IFNγ (25-27). In contrast, PGE_2, depending on stimulatory conditions, either has no effect or enhances production of Th2 cytokines, such as IL-4, IL-5 and IL-10 (26-28). We examined the hypothesis that increases in splenic PGE_2-MØ following high dose BCG immunization promote the Th1-to-Th2 shifts of immune responses.

METHODS AND MATERIALS

Mice. Non-pregnant female C57Bl/6 and Balb/c mice, 8-14 weeks old, from the Jackson Laboratory (Bar Harbor, ME) were used. Mice were maintained in barrier-filtered cages and fed Purina laboratory chow and tap water *ad libitum*. Experimental protocols employed in this study were approved by IACUC of Brody School of Medicine at East Carolina University.

Preparations of HK-BCG. As described previously (6), the cultured bacteria of *M. bovis* Calmette-Guerin bacillus (BCG) Tokyo 172 strain were washed, autoclaved, and lyophilized. The powder of HK-BCG was suspended in saline immediately before use. The suspensions of HK-BCG were dispersed by brief (10s) sonication prior to injection. These HK-BCG preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the *Limulus* amebocyte lysate assay (Sigma, St. Louis, MO) (29).

Immunization of mice with HK-BCG. Groups of mice received i.p. doses (0.01 and 1 mg/dose) of HK-BCG (4 - 5 mice/group) on day 0. Controls received 0.2 ml of saline. In some experiments, BCG-immunized mice and their controls were further treated with nimesulide, nimflumic acid, and indomethacin (all from Cayman, Ann Arbor, MI) all at 50 mg/kg i.p., daily, starting on day 1. Control groups of mice received 0.5% ethanol in saline (0.2 ml/dose). On day 14, spleens and sera were harvested.

Cytokine production in recall response of spleen cell cultures. Spleens from each group of mice were isolated and pooled, minced with scissors, and digested with collagenase D (C2139, Sigma) at 50 U/ml in RPMI 1640 plus 10% FBS at 37°C for 60 min and filtered through a 100-μm

mesh. Single cell suspensions were prepared by washing digested cells with RPMI 1640 containing DNase at 100 μg/ml (DN-25, Sigma). After washing with serum-free RPMI 1640, cell suspensions were applied to the top of a discontinuous Percoll gradient (35/60%). Following centrifugation, 800 g, 30 min, 22°C, cells between 35% and 60% Percoll were collected. Spleen cells were suspended in RPMI 1640 plus 10% FBS at 4 x 10^6 cells/ml and incubated with endotoxin-free mycobacterial HSP65 (Stressgen, Victoria, BC Canada) and purified protein derivative (PPD, Japan BCG Laboratory) at 5 and 50 μg/ml, respectively, for 4 days. In some experiments, nimesulide was added at 10^{-6} M to the cultures. After incubation, culture supernatants were collected and their IL-4, IL-10 and IFNγ levels were measured by the respective specific ELISA (PharMingen, San Diego, CA).

PGE_2-MØ. Plastic adherent splenic MØ were isolated from spleen cell suspensions prepared above (18,19). Splenic MØ (2 x 10^6 cells/ml) were cultured in serum-free RPMI 1640 medium with either 10^{-6} M calcium ionophore A23187 (Sigma), 1μg/ml arachidonic acid (AA, Cayman), or 1 μg/ml bacterial endotoxin (LPS, Sigma) for 2 hrs. In some experiments, splenic cells (2 x 10^6 cells/ml) were cultured in RPMI 1640 medium plus 2% FBS with 50 μg/ml PPD for 2 days in the presence of 1 μM nimesulide, indomethacin, or NS-398. PGE_2 levels in the culture supernatants were measured by a competitive ELISA (Cayman).

Prostaglandin E synthase (PGES) assay. PGES activities in cell lysates were measured by assessment of conversion of PGH_2 to PGE_2 (30). Adherent splenic MØ in 400 μl 10 mM Tris buffer, pH 8.0, were disrupted by sonication using a Branson Sonifer (10s, three times, 1-min interval). After centrifugation of the sonicates at 1,700 g for 10 min, 4°C, the supernatants were

used as the enzyme source. An aliquot of each lysate (10 μg protein) was incubated with 0.5 μg of PGH_2 (Cayman) for 30 s at 24°C in 0.1 ml 0.1 M Tris buffer, pH 8.0, containing 1 mM reduced L-glutathione (Sigma) and 5 μg of indomethacin. After terminating the reaction by the addition of 100 mM $FeCl_2$, PGE_2 in the supernatants was quantified with the PGE_2 EXPRESS EIA kit (Cayman). Protein concentrations were determined by bicinchoninic acid assay kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

Total serum IgE, antigen-specific IgG1 and IgG2a. Total serum IgE levels was determined by ELISA using purified mouse IgE kappa isotype as a standard, rat anti-mouse IgE monoclonal antibody (clone R35-72) as a capture antibody, and biotinylated rat monoclonal antibodies detecting IgE (clone R25-92) as a detection antibody (all reagents from PharMingen). Levels of PPD-specific IgG1/IgG2a and HSP65-specific IgG1 and IgG2a were measured by ELISA with 96-well plates coated overnight at 4°C with PPD and HSP65 at 0.5 μg/0.1 ml/well and 0.1 μg/0.1 ml/well, respectively, in 0.05 M sodium carbonate buffer, pH 9.6.. Biotinylated rat monoclonal antibodies detecting IgG1 and IgG2a were clones A85-1 and R19-15, respectively (PharMingen).

Cytometric detection. The expression of surface antigens on the spleen cell preparations was determined by indirect immunofluorescence in the presence of 5% heat –inactivated newborn calf serum (Sigma), pH 7.2 (31). Rat monoclonal antibodies used for the analyses were Mac-1 (β2 integrin), RB6-8C5 (neutrophil), MOMA-1 (marginal zone metalophilic MØ) and ER-TR9 (marginal zone MØ) (all from Research Diagnostics, Flanders, NJ). Fluorescein isothiocyanate-conjugated donkey anti-rat IgG (Jackson ImmunoResearch, West Glove, PA) was used as a secondary antibody for indirect immunofluorescence.

To determine expression of cytosolic antigens, spleen cells prepared above were fixed with 4% paraformaldehyde, permeabilized with 1% saponin, and stained with rabbit antibodies specific for PGHS-1, PGHS-2, and mPGES at 2 μg/ml (Cayman) (31). The presence of the primary antibody was determined by FITC-conjugated donkey anti-rabbit IgG at 1:1,000 dilution (Jackson ImmunoResearch). Cytosolic PGES (cPGES) was detected with monoclonal anti-cPGES (anti-p23, Clone JJ3, Alexis Biochemicals, San Diego, CA) and FITC-conjugated donkey anti-murine IgG at 1:1,000 as secondary antibody (Jackson ImmunoResearch).

Fluorescence of 10^4 stained cells was quantitated with a FACScan flow cytometer using the CellQuest program (Becton Dickinson, Mountain View, CA). All cells as defined by forward and sideward scatter pattern were gated; only debris was excluded from analysis. Cells stained with the second antibody alone were used as negative controls in all experiments.

Statistics. Data were analyzed by one way analysis of variance. For cell culture studies, tissues isolated from at least 5 mice were pooled unless indicated; cells were cultured in at least triplicate in each group. *P* value of less than 0.05 is considered statistically significant.

RESULTS

HK-BCG induces splenic PGE_2-MØ in Balb/c mice. Splenic MØ isolated from normal mice show minimum levels of PGE_2 release (18,19). To induce splenic PGE_2-MØ and determine whether these cells contribute to the Th1-to-Th2 shift of immune responses, Balb/c mice were immunized intraperitoneally with 0.01 or 1 mg HK-BCG. When splenic MØ were isolated 1 to 3 days after immunization with 1 mg HK-BCG and stimulated *in vitro* with A23187 for 2 hrs, PGE_2 release was not different from control cells obtained from unimmunized animals (Figure 1). Significantly higher PGE_2 release was observed 14 days after immunization with 1 mg HK-BCG (Figure 1A). Furthermore, free AA, but not LPS, also elicited PGE_2 release (Figure 1A). Shown in Figure 1B PPD, mycobacterial antigens, induced spleen cells to release PGE_2 suggesting that interaction between antigen-specific lymphocytes and splenic MØ triggers PGE_2 biosynthesis. Furthermore, this PGE_2 biosynthesis was inhibited by N-398, nimesulide or indomethacin (Figure 1B).

Cytometric detection of PGHS-1$^+$, PGHS-2$^+$, mPGES$^+$, and cPGES$^+$ spleen cells in Balb/c mice. At day 14 after immunization with 1 mg BCG, Balb/c mice showed significant splenomegaly and an increase in MØ expressing Mac-1 (β2 integrin), RB6-8C5 (neutrophil) and MOMA-1 (marginal zone metalophilic MØ), but not ER-TR9 (marginal zone MØ) (Figure 2A). However, these cells were not increased by immunization with 0.01 mg HK-BCG.

PGE_2-MØ metabolize endogenous AA to PGE_2 through the rate-limiting enzymes PGH synthase (PGHS) and PGE synthase (PGES). Two major isoforms of PGHS convert AA to PGH_2: PGHS-1,

a constitutive form; and PGHS-2, an inducible form that appears to be up-regulated in response to HK-BCG immunization as well as LPS and cytokines (Manuscript in preparation). PGH_2 is further converted to PGE_2 by cytosolic PGES (cPGES) and microsomal PGES (mPGES) (30,32). As shown in Figure 2B, normal splenic MØ expressed PGHS-1, mPGES and cPGES but not PGHS-2. This profile was not changed from the unimmunized control animals following immunization with 0.01 mg HK-BCG. When immunized with 1 mg HK-BCG, however, not only PGHS-1^+, mPGES$^+$ and cPGES$^+$ cells but also PGHS-2^+ cells were increased on day 14 (Figure 2B).

PGES activities. Cytosolic and membrane-bound glutathione-dependent PGES (cPGES and mPGES) have been shown to be terminal enzymes of the PGHS-1 and PGHS-2-mediated PGE_2 biosynthesis, respectively (30,33). Cells expressing either mPGES$^+$ or cPGES$^+$ were both increased when mice were immunized with 1 mg HK-BCG (Figure 2B). We further determined whether cellular PGES activity was increased by immunization with 1 mg HK-BCG. As shown in Figure 3, the PGES activity was not significantly altered by immunization with HK-BCG.

Serum IgE, IgG1 and IgG2a in Blab/c mice. Endogenous Th2 and Th1 cytokines are isotype-switching signals for antigen-specific B cells, which bias the serum IgE/IgG1and IgG2a, respectively (34,35). We observed that immunization of Balb/c mice with 1 mg of HK-BCG resulted in increased levels of total IgE, HSP65-specific IgG1 and HSP65-specific IgG2a (Figure 4). In contrast, when immunized with 0.01 mg of HK-BCG, there was no increase in total serum IgE or HSP65-specific IgG1, but a significant increase in HSP65-specific IgG2a (Figure 4). Although immunization with 1mg HK-BCG resulted in increases in both PPD-specific IgG1 and

IgG2a, there was no PPD-specific IgG1 or IgG2a detected in mice that were immunized with 0.01 mg HK-BCG. Our results suggest that HK-BCG at 0.01 mg induces IgG2a production. However, 1 mg of HK-BCG induces higher levels of IgG1 and IgE production in addition to IgG2a production indicating a switch to a Th2 response.

Splenic Th1 and Th2 immune responses against mycobacterial antigens in Balb/c mice. To further determine whether immunization with 1 mg HK-BCG induces a mixed Th1 and Th2 response, selected cytokine levels specific for Th1 or Th2 cells were measured in recall responses of spleen cell cultures. When spleen cells from Balb/c mice immunized with 0.01 mg HK-BCG were stimulated *in vitro* with 50 μg/ml PPD or 5 μg/ml HSP65, relatively large amounts of IFNγ were detected (Figure 5). The spleen cells, however, did not produce IL-10 (Figure 5), IL-4 or IL-5 (data not shown). In contrast, immunization with 1 mg of HK-BCG resulted in the production of both IFNγ and IL-10 (Figure 5). Neither IL-4 nor IL-5 was detected (data not shown). Interestingly, HSP65 did not stimulate IL-10 production, suggesting antigen-specificity in the Th2 responses on day 14. When spleen cell cultures in recall responses were treated with 1 μM nimesulide, a PGHS-2 inhibitor, IFNγ production but not IL-10 production was significantly enhanced, suggesting consistent with a PGE_2-induced shift from Th1 to Th2 response.

High doses of BCG induce splenic PGE_2-M$\varnothing$ and Th1-to-Th2 shifts in C57Bl/6 mice. Genetic background in C57Bl/6 and Balb/c mice is important in the balance of Th1/Th2 differentiation (36-38). Splenic M$\varnothing$ from C57Bl/6 released lower levels of PGE_2 *in vitro* than those from Balb/c mice (39). To assess whether immunization of C57Bl/6 mice with HK-BCG induces a Th1-to-Th2 shift, 0.01 or 1.0 mg HK-BCG was administered intraperitoneally to the

mice. As shown in Figure 6, immunization with HK-BCG resulted in a dose dependent increase in splenic PGE_2-M∅ activity, but the amounts of PGE_2 released were significantly lower than those in Balb/c mice (Figures 1 and 6). Spleen cells from C57Bl/6 mice immunized with 1.0 mg HK-BCG and cultured with 50 μg/ml PPD in a recall response also released a relatively high level of PGE_2, a low level of IFNγ, and increased IL-4 compared to cells from mice immunized with HK-BCG at 0.01mg (Figure 6). Unlike Balb/c mice (Figure 5), 1 mg HK-BCG in C57Bl/6 mice induced IL-4 production (Figure 6), but not detectable levels of IL-10 (data not shown). Similarly to Balb/c mice, immunization of C57Bl/6 mice with 1 mg HK-BCG induced significantly higher levels of serum IgE than that with 0.01 mg HK-BCG or saline (Figure 6).

PGHS inhibitors inhibit Th2, but enhance Th1 responses in C57Bl/6 mice. To assess the role of PGE_2 in the shift from Th1-to-Th2 response, HK-BCG-injected mice were treated with nimesulide and niflumic acid, PGHS-2 inhibitors. Figure 7 shows that the treatments resulted in the production of more IFNγ but less IL-4 by PPD-stimulated spleen cells. Similarly, indomethacin, a PGHS-1 inhibitor, is also effective. These results suggest that PGE_2-M∅ induce the shift from Th1-to-Th2 response in HK-BCG-treated mice and that PGE_2 is synthesized through both PGHS-1 and PGHS-2.

DISCUSSION

Live BCG induces not only host resistance against *M. tuberculosis* but also tumoricidal activity in the host and has been widely used for treatment of superficial bladder cancer (7,40). Live BCG also induces a non-specific microbicidal MØ activity against *Listeria monocytogenes* (41,42) and *Toxoplasma gondii* (43). The effects, however, are inconsistent probably due to various host responses that alter bacterial growth and production of bioactive components (44). To understand the interactions between hosts and bioactive mycobacterial components, we employed selected doses of HK-BCG. A low dose (0.01 mg) of HK-BCG induced a Th1 lymphocyte response against mycobacteria, although the effects are not enough to protect against tuberculosis (45). In sharp contrast, 1 mg doses of HK-BCG induced increases in the total serum IgE level, as well as serum IgG1 and IgG2a levels against mycobacterial antigens, indicating a mixture of Th1 and Th2 immune responses. In this regard, the recall responses in cultured spleen cells exhibit typical Th1 and Th2 cytokine production in responding to mycobacterial antigens (PPD) (Figure 5). Although HSP65-specific IgG1 levels were high (Figure 4), there was no Th2 cytokine production to HSP65 in the recall response. Since endogenous antigen profiles would be distinct in mice challenged with live BCG or HK-BCG, our model cannot, therefore, provide evidence of whether the magnitude of the Th2 response is antigen–dependent or -independent (13,17).

Heat-resistant cell wall mycoloyl glycolipids in *Mycobacterium bovis* BCG and *Corynebacterium parvum* (*Propionibacterium acnes*) are known to induce PGE_2-MØ (46). Previous studies clearly indicate that immunization of mice with HK-BCG and HK-*C. parvum* results in the formation of splenic PGE_2-MØ 5 – 21 days after immunization in a dose-dependent manner (6,18). Splenic

PGE_2-MØ express Fcγ receptors and phagocytic activity (18). In this study, 1 mg HK-BCG, but not 0.01 mg, induced splenic PGE_2-MØ, on day 14. Splenic PGE_2-MØ release >10 ng $PGE_2/10^6$ MØ in Balb/c mice when elicited *in vitro* with calcium ionophore and AA, but not LPS. Since both recall responses in cultured splenic MØ and ionophore-stimulated cultured cells release relatively large amounts of PGE_2 (Figures 2 & 6), splenic PGE_2-MØ would appear to release PGE_2 locally during immunization with HK-BCG.

Splenic PGE_2-MØ formation, therefore, is associated with the induction of Th1-to-Th2 shifts of lymphocyte responses. This is further demonstrated by the finding that the inhibition of splenic PGE_2-MØ activities *in vitro* or *in vivo* by nimesulide, niflumic acid or indomethacin, results in recovery of a Th1-dominant phenotype with decreased Th2 responses (Figures 5 & 7). Since these inhibitors act on both PGHS-1 and PGHS-2, our study cannot determine which is more important, PGHS-1 or PGHS-2, for PGE_2 synthesis. Furthermore, our results cannot rule out the contribution of PGHS-3, a recently identified PGHS-1 variant (47). In addition, recent studies indicate that pharmacological effects of indomethacin include more than the inhibition of PGHS, directly regulating Th2 cells through modification of a PGD_2 receptor subtype (48).

PGE_2 synthesis in splenic PGE_2-MØ is dependent on increases in cellular calcium levels that appear to activate phospholipase A_2 (PLA_2). Exogenous free AA bypasses the PLA_2 pathway(s). However, our results (Figure 1) clearly indicate that, unlike other MØ populations such as peritoneal MØ, PGE_2 synthesis in splenic PGE_2-MØ is not elicited by LPS (49). Since LPS induces PGHS-2 in splenic MØ *in vitro* (36), there appears to be a significant gap between high PGHS-2 levels and PGE_2 synthesis in splenic MØ. In this connection, LPS as well as HK-BCG

could induce both cPGES and mPGES in splenic MØ *in vitro* (30,33) and *in vivo* (Figure 2), respectively. However, cellular PGES activities in normal splenic MØ are as high as in splenic PGE_2-MØ (Figure 3). Therefore, the regulatory mechanisms of PGE_2 synthesis in splenic PGE_2-MØ are complex and additional factors must contribute to the enhanced PGE_2 formation by splenic PGE_2-MØ isolated from mice immunized with high doses of HK-BCG. Secretory PLA_2 type V is known to be involved in prostaglandin production in splenic MØ, mast cells and mesangial cells (36,51,52). Further biochemical analyses to identify rate-limiting factor(s) of PGE_2 synthesis are required in splenic PGE_2-MØ.

How do high doses of BCG induce the formation of PGE_2-MØ in the spleen? Although inflammatory mediators including bacterial endotoxin directly prime normal splenic MØ to enhance PGE_2-releasing capacity, the PGE_2 levels are far smaller than those released by splenic PGE_2-MØ (manuscript in preparation). Previously we found that splenic PGE_2-MØ are not formed following immunization with HK-*C. parvum* in mice treated with a bone-seeking isotope, ^{89}Sr to deplete bone marrow cells. These mice, however, show increases in total splenic MØ (18,19,52). It is likely, therefore, that PGE_2-MØ precursors are generated in the bone marrow following mycobacterial inoculation, then migrate, and localize in the spleen where antigen-specific Th1/Th2 lymphocytes are activated. Therefore, splenic PGE_2-MØ are strategically located to induce the shift of Th1-to-Th2 response in the spleen during mycobacterial infections. Once PGE_2-MØ are established, they persist for long periods (18) and therefore, the effect of PGE_2 on immune regulation is prolonged. Splenic Th1/Th2 cells eventually migrate to inflammatory sites (8). It should also be noted that splenic PGE_2-MØ formation is independent of circulating monocytes (52).

Balb/c mice, compared to C57Bl/6 mice, frequently show the induction of Th2 responses after infection or immunization, such as BCG (8) and *Leishmania major* (53). One of the mechanisms to induce Th2 response in Balb/c mice is the participation of IL-4, which is rapidly and abundantly produced by *L. major* infection and induces selective loss of IL-12 receptor expression and IFNγ production (54). Kuroda and Yamashita (36,55) showed that normal splenic MØ isolated from Balb/c mice treated *in vitro* with bacterial endotoxin, produce more PGE_2 than those from C57Bl/6 mice, and have a greater sensitivity to the suppressive effect of PGE_2. Although our study supports such distinction, we found that both strains of mice showed a Th1-to-Th2 shift of immune response against mycobacteria when responding to high doses of HK-BCG.

In this regard, Th2 cytokine profiles in the *in vitro* recall responses appear to be distinct between Balb/c and C57Bl/6, which predominantly produce IL-10 and IL-4, respectively, when immunized with high doses of HK-BCG. Recently, a new group of $CD4^+$ T cells, T regulatory cells, have been found to suppress the development of autoaggressive immune responses in a cell-to-cell contact manner (56). IL-10 is produced by T regulatory cells and differentiates T regulatory cells in auto- and para-crine manners (56). It remains to be elucidated whether T regulatory cells are involved in the induction and/or maintenance of the Th1-to-Th2 shift of immune response in either or both strains of mice following immunization with high doses of HK-BCG.

In conclusion, previously we found that development of splenic PGE_2-MØ are dependent on and probably derived from bone marrow cells (18,19,52). We re-visited the unique splenic PGE_2-MØ population that plays a novel immunoregulatory role in the Th1-to-Th2 shift of immune responses

against mycobacterial antigens including HSP65. Further study of mechanisms underlying formation of PGE_2-MØ should provide the basis for pharmacologically targeting the formation of splenic PGE_2-MØ and the resultant Th1-to-Th2 shift of the immunologic response. Finally, it remains to be elucidated whether similar mechanisms are involved in the pathogenesis of cancer (23,57), *Leishmania* infection, *Trichophyton* dermatophytosis, syphilitic infection, and HIV infection progressing to AIDS (28,53,58,59) where Th1-to-Th2 shifts of immune responses and splenic PGE_2-MØ formation are frequently seen. Such shifts are also observed in neonatal animals, aged animals, and tumor-bearing animals, animals with diabetes and animals with hypercholesterolemia (21,60-62).

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REFERENCES

1. Yong, A. J., J. M. Grange, R. D. Tee, J. S. Beck, G. H. Bothamley, D. M. Kemeny, T. Kardjito. 1989. Total and anti-mycobacterial IgE levels in serum from patients with tuberculosis and leprosy. *Tubercle.* 70:273.

2. Surcel, H. M., M. Troye-Blomberg, S. Paulie, G. Andersson, C. Moreno, G. Pasvol, J. Ivanyi. 1994. Th1/Th2 profiles in tuberculosis, based on the proliferation and cytokine response of blood lymphocytes to mycobacterial antigens. *Immunology* 81:171.

3. Yamamura, M., K. Uyemura, R. J. Deans, K. Weinberg, T. H. Rea, B.R. Bloom, R.L. Modlin. 1991. Defining protective responses to pathogens: cytokine profiles in leprosy lesions. *Science* 254:277.

4. Peason, C. M. 1956. Development of arthritis, periarthritis and periostitis in rats given adjuvants. *Proc. Soc. Exp. Biol. Med.* 91:95.

5. Matthys, P., K. Vermeire, A. Billiau. 2001. Mac-1(+) myelopoiesis induced by CFA: a clue to the paradoxical effects of IFN-gamma in autoimmune disease models. *Trends Immunol.* 22:367.

6. Shibata, Y., I. Honda, J. P. Justice, M. R. van Scott, R. M. Nakamura, Q. N. Myrvik. 2001. A Th1-adjuvant --- N-acetyl-D-glucosamine polymer up-regulates Th-1 immunity but down-regulates Th-2 immunity against a mycobacterial protein (MPB-59) in IL-10-knockout and wild type mice. *Infect. Immun.* 69:6123.

7. Amling, C. L. 2001. Diagnosis and management of superficial bladder cancer. *Curr. Probl. Cancer* 25:219.

8. Power CA, Wei G, Bretscher PA. 1998. Mycobacterial dose defines the Th1/Th2 nature of the immune response independently of whether immunization is administered by the intravenous, subcutaneous, or intradermal route. *Infect. Immun.* 66:5743.

9. Hsieh, C. S., A. B. Heimberger, J. S. Gold, A. O'Garra, K. M. Murphy. 1992. Differential regulation of T helper phenotype development by interleukins 4 and 10 in an alpha beta T-cell-receptor transgenic system. *Proc. Natl. Acad. Sci. U. S. A.* 89:6065.

10. Oswald, I. P., R. T. Gazzinelli, A. Sher, S. L. James. 1992. IL-10 synergizes with IL-4 and transforming growth factor-beta to inhibit macrophage cytotoxic activity. *J. Immunol.* 148:3578.

11. Marone, G., S. J. Galli, Y. Kitamura. 2002. Probing the roles of mast cells and basophils in natural and acquired immunity, physiology and disease. *Trends Immunol.* 23:425.

12. Lowrie, D. B., R. E. Tascon, V. L. Bonato, V. M. Lima, L. H. Faccioli, E. Stavropoulos, M. J. Colston, R. G. Hewinson, K. Moelling, C. L. Silva. 1999. Therapy of tuberculosis in mice by DNA vaccination. *Nature* 400:269.

13. Orme, I. M., A. D. Roberts, J. P. Griffin, J. S. Abrams. 1993. Cytokine secretion by CD4 T lymphocytes acquired in response to Mycobacterium tuberculosis infection. *J. Immunol.* 151:518

14. Mayr, M., S. Kiechl, J. Willeit, G. Wick, Q. Xu. 2000. Infections, immunity, and atherosclerosis: associations of antibodies to *Chlamydia pneumoniae*, *Helicobacter pylori*, and cytomegalovirus with immune reactions to heat-shock protein 60 and carotid or femoral atherosclerosis. *Circulation* 102:833.

15. Hernandez-Pando R, Orozcoe H, Sampieri A, Pavon L, Velasquillo C, Larriva-Sahd J, Alcocer JM, Madrid MV. 1996. Correlation between the kinetics of Th1, Th2 cells and pathology in a murine model of experimental pulmonary tuberculosis. *Immunology* 89:26.

16. Jung, Y. J., R. LaCourse, L. Ryan, R. J. North. 2002. Evidence inconsistent with a negative influence of T helper 2 cells on protection afforded by a dominant T helper 1 response against Mycobacterium tuberculosis lung infection in mice. *Infect Immun.* 70:6436.

17. Jiao, X., R. Lo-Man, N. Winter, E. Deriaud, B. Gicquel, C. Leclerc. 2003. The shift of Th1 to

Th2 immunodominance associated with the chronicity of *Mycobacterium bovis* bacille Calmette-Guerin infection does not affect the memory response. *J. Immunol.* 170:1392.

18. Shibata, Y., A. Volkman. 1985. The effect of bone marrow depletion on prostaglandin E producing suppressor macrophages in mouse spleen. *J. Immunol.* 135:3897.

19. Shibata, Y., A. P. Bautista, S. N. Pennington, J. L. Humes, A. Volkman. 1987. Eicosanoid production by peritoneal and splenic macrophages in mice depleted of bone marrow by 89Sr. *Am. J. Pathol.* 127:75.

20. Fitzgerald, T. J. 1992. The Th1/Th2-like switch in syphilitic infection: is it detrimental? *Infect. Immun.* 60:3475.

21. Snyder, D. S., C. Y. Lu, E. R. Unanue. 1982. Control of macrophage Ia expression in neonatal mice--role of a splenic suppressor cell. *J. Immunol.* 128:1458.

22. Reiner, N. E., W. Ng, W. R. McMaster. 1987. Parasite-accessory cell interactions in murine leishmaniasis. II. Leishmania donovani suppresses macrophage expression of class I and class II major histocompatibility complex gene products. *J. Immunol.* 138:1926.

23. Oghiso, Y., Y. Yamada, K. Ando, H. Ishihara, Y. Shibata. 1993. Differential induction of prostaglandin E2-dependent and -independent immune suppressor cells by tumor-derived GM-CSF and M-CSF. *J. Leukoc. Biol.* 53:86.

24. Hayek, M. G., S. N. Meydani, M. Meydani, J. B. Blumberg. 1994. Age differences in eicosanoid production of mouse splenocytes: effects on mitogen-induced T-cell proliferation. *J. Gerontol.* 49:B197.

25. Betz, M., B. S. Fox.1991. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. *J. Immunol.* 146:108.

26. Snijdewint, F. G., P. Kalinski, E. A. Wierenga, J. D. Bos, M. L. Kapsenberg. 1993.

Prostaglandin E2 differentially modulates cytokine secretion profiles of human T helper lymphocytes. *J. Immunol.* 150:5321.

27. Rocca, B., L. M. Spain, E. Pure, R. Langenbach, C. Patrono, G. A. FitzGerald. 1999. Distinct roles of prostaglandin H synthases 1 and 2 in T-cell development. *J. Clin. Invest.* 103:1469.

28. Fitzgerald, T. J. 1992. The Th1/Th2 switch in syphilitic infection: is it detrimental? *Infect. Immun.* 60:3475.

29. Shibata, Y., L. A. Foster, H. Okamura, R. M. Nakamura, et al. 1998. Immuno-regulatory roles of IL-10 in innate immunity-IL-10 inhibits macrophage production of IFNγ-inducing factors but enhances NK cell production of IFNγ. *J. Immunol.* 161:4283.

30. Murakami, M., H. Naraba, T. Tanioka, N. Semmyo, Y. Nakatani, F. Kojima, T. Ikeda, M. Fueki, A. Ueno, S. Oh, I. Kudo. 2002. Regulation of prostaglandin E2 biosynthesis by inducible membrane-associated prostaglandin E2 synthase that acts in concert with cyclooxygenase-2. *J. Biol. Chem.* 275:32783.

31. Shibata. Y., D. R. Bjorkman, M. Schmidt, Y. Oghiso, A. Volkman. 1994. Macrophage colony-stimulating factor-induced bone marrow macrophages do not synthesize or release prostaglandin E2. *Blood* 83:3316.

32. Uematsu, S., M. Matsumoto, K. Takeda, S. Akira. 2000. Lipopolysaccharide-dependent prostaglandin E(2) production is regulated by the glutathione-dependent prostaglandin E(2) synthase gene induced by the Toll-like receptor 4/MyD88/NF-IL6 pathway. *J. Immunol.* 168:5811.

33. Tanioka, T., Y. Nakatani, N. Semmyo, M. Murakami, I. Kudo. 2000. Molecular identification of cytosolic prostaglandin E2 synthase that is functionally coupled with cyclooxygenase-1 in immediate prostaglandin E2 biosynthesis. *J. Biol. Chem.* 275:32775.

34. Coffman, R. L., J. Ohara, M. W. Bond, J. Carty, A. Zlotnik, W. E. Paul. 1986. B cell stimulatory factor-1 enhances the IgE response of lipopolysaccharide-activated B cells. *J. Immunol.* 136:4538.

35. Snapper, C. M., W. E. Paul. 1987. Interferon-gamma and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. *Science* 236:944.

36. Kuroda, E., U. Yamashita. 2003. Mechanisms of enhanced macrophage-mediated prostaglandin E2 production and its suppressive role in Th1 activation in Th2-dominant Balb/c mice. *J. Immunol.* 170:757.

37. Hoft, D. F., R. G. Lynch, L. V. Kinchhoff. 1993. Kinetic analysis of antigen-specific immune responses in resistant and susceptible mice during infection with *Trypanosoma cruzi. J. Immunol.* 151:7038.

38. Autenrieth, I. B., M. Beer, E. Bohn, S. H. E. Kaufmann, J. Heesemann. 1994. Immune responses to *Yersinia enterocolitica* in susceptible Balb/c and resistance C57Bl/6 mice: an essential role for γ-interferon. *Infect. Immun.* 62:2590.

39. Keane-Myers, A., S. P. Nickell. 1995. Role of IL-4 and IFN-γ in modulation of immunity to *Borrelia burgdorferi* in mice. *J. Immunol.* 155:2020.

40. Kuroda, K., E. J. Brown, W. B. Telle, D. G. Russell, T. L. Ratliff. 1993. Characterization of the internalization of bacillus Calmette-Guerin by human bladder tumor cells. *J. Clin. Invest.* 91:69.

41. Simon, H. B., J. N. Sheagren. 1971. Cellular immunity in vitro. I. Immunologically mediated

enhancement of macrophage bactericidal capacity. *J. Exp. Med.* 133:1377.

42. Leal, I. S., R. Appelberg, M. T. Silva. 1996. Neutrophils are involved in the non-specific resistance to listeriosis induced by mycobacterial infections. *Immunol.* 89:442.

43. van Dissel, J. T., J. J. Stikkelbroeck, M. T. van den Barselaar, W. Sluiter, P. C. Leijh, R. van Furth. 1987. Divergent changes in antimicrobial activity after immunologic activation of mouse peritoneal macrophages. *J. Immunol.* 139:1665.

44. Wing, E. J. 1981. Bacillus Calmette-Guerin (BCG) decreases resistance to *Listeria monocytogenes* infection in mice. *Immunol.* 44:509.

45. McDonough, K. A., Y. Kress, B. R. Bloom. 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. *Infect. Immun.* 61:2763.

46. Han, Y., N. Kawada, I. Yano. 1998. Granuloma formation and in vitro macrophage activation in mice by mycoloyl glycolipids from Norardia asteroids and related taxa. *Osaka City Med. J.* 44:201.

47. Chandrasekharan, N. V., H. Dai, K. L. Roos, N. K. Evanson, J. Tomsik, T. S. Elton, D. L. Simmons. 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci. U. S. A.* 99:13926.

48. Hirai, H., K. Tanaka, S. Takano, M. Ichimasa, M. Nakamura, K. Nagata. 2002. Cutting edge: agonistic effect of indomethacin on a prostaglandin D2 receptor, CRTH2. *J. Immunol.* 168:981.

49. Aderem, A. A., D. S. Cohen, S. D. Wright, Z. A. Cohn. 1988. Bacterial lipopolysaccharides prime macrophages for enhanced release of arachidonic acid metabolites. *J. Exp. Med.* 164:165.

50. Reddy, S. T., M. V. Winstead, J. A. Tischfield, H. R. Herschman. 1997. Analysis of the secretory phospholipase A2 that mediates prostaglandin production in mast cells. *J. Biol. Chem.*

272:13591.

51. Han, W. K., A. Sapirstein, C. C. Hung, A. Alessandrini, J. V.Bonventre. 2003. Cross-talk between cytosolic phospholipase A2 alpha (cPLA2 alpha) and secretory phospholipase A2 (sPLA2) in hydrogen peroxide-induced arachidonic acid release in murine mesangial cells: sPLA2 regulates cPLA2 alpha activity that is responsible for arachidonic acid release. J. *Biol. Chem.* 278:24153.

52. Shibata, Y. 1989. Restoration of prostaglandin E2-producing splenic macrophages in 89Sr-treated mice with bone marrow from *Corynebacterium parvum* primed donors. *Reg. Immunol.* 2:169.

53. Bretscher, P. A., G. Wie, J. N. Menon, H. Bielefeldt-Ohmann. 1992. Establishment of stable, cell-mediated immunity that makes "susceptible" mice resistant to *Leishmania major. Science* 257:539.

54. Guler, M. L., J. D. Gorham, C. S. Hsieh, A. J. Mackey, R. G. Steen, W. F. Dietrich, K. M. Murphy. 1996. Genetic susceptibility to Leishmania: IL-12 responsiveness in TH1 cell development. *Science* 271:984.

55. Kuroda, E., T. Sugiura, K. Zeki, Y. Yoshida, U. Yamashita. 2000. Sensitivity difference to the suppressive effect of prostaglandin E2 among mouse strains: a possible mechanism to polarize Th2 type response in BALB/c mice. *J. Immunol.* 164:2386.

56. Jonuleit, H., E. Schmitt. 2003. The regulatory T cell family: distinct subsets and their interrelations. *J. Immunol.* 171:6323.

57. Druker, B. J., H. T. Wepsic. 1983. BCG-induced macrophages as suppressor cells. *Cancer Investig.* 1:151.

58. Woodfolk, J. A., J. B. Slunt, B. Deuell, M. L. Hayden, T. A. E. Platts-Mills. 1996. Definition

of Trichophyton protein associated with delayed hypersensitivity in humans. Evidence for immediate (IgE and IgG4) and delayed hypersensitivity to a single protein. *J. Immunol.* 156:1695.

59. Salk, J., P. A. Bretcher, P. L. Salk, M. Clerici, G. M. Shearer. 1993. A strategy for prophylactic vaccination against HIV. *Science* 260:1270.

60. Zhou, X., G. Paulsson, S. Stemme, G. K. Hansson. 1998. Hypercholesterolemia is associated with a T helper (Th) 1/Th2 switch of the autoimmune response in atherosclerotic apo E-knockout mice. *J. Clin. Invest.* 15:1717.

61. Serreze, D. V., H. D. Chapman, C. M. Post, E. A. Johnson, W. L. Suarez-Pinzon, A. Rabinovitch. 2001. Th1 to Th2 cytokine shifts in nonobese diabetic mice: sometimes an outcome, rather than the cause, of diabetes resistance elicited by immunostimulation. *J. Immunol.* 166:1352.

62. Ginaldi, L., M. De Martinis, A. D'Ostilio, L. Marini, M. F. Loreto, D. Quaglino. 1999. The immune system in the elderly: III. Innate immunity. *Immunol. Res.* 20:117.

Footnote

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Abbreviations used in this paper: MØ, macrophages; CMI, cell-mediated immunity; BCG, Bacillus Calmette-Guérin; PGE_2, prostaglandin E_2; HK, heat killed; PGHS, prostaglandin G/H synthase; PGES, prostaglandin E synthase; HSP, heat shock protein; PPD, purified protein derivative

Legends for Figures

Figure 1. HK-BCG induces splenic PGE_2-MØ formation in Balb/c mice. Groups of Balb/c mice received i.p. doses (0.01 and 1 mg/dose) of HK-BCG (4 mice/group) on day 0. Day 0 (untreated), day 1, day 3 and day 14, spleens were harvested. (A) Splenic MØ were isolated, pooled in each group, incubated in serum-free RPMI 1640 medium (10^6 MØ/ml) in the presence of AA at 1 μg/ml, A23187 at 1 mM, LPS at 1 μg/ml or media alone for 2 hrs. (B) Spleen cells were incubated in RPMI 1640 plus 2% FBS (2×10^6 cells/ml) in the presence of PPD at 50 μg/ml mixed with or without nimesulide at 1 μM for 48 hrs. The levels of PGE_2 were measured by ELISA. Mean ± SD, n=3. Each result represents a group of 4 mice from three experiments.

Figure 2. PGHS-1^+ and PGHS-2^+ spleen cells were increased in HK-BCG-treated Balb/c mice. Groups of Balb/c mice received i.p. doses (0.01 [open] and 1 mg/dose [close]) of HK-BCG (5 mice/group) on day 0. Day 0 (untreated), day 1, day 3 and day 14, spleens were harvested. Nucleated spleen cells were isolated, pooled in each group and analyzed by flow cytometry. Each result represents a group of 4 mice from three experiments. (●○), total nucleated spleen cells per mouse [or per group??]; expression of Mac-1 (▲△), RB6-8C5 (▼▽), MOMA-1 (◆◇) and ER-TR9 (■□) on the plasma membrane (A). Expression of intracellular PGHS-1 (●○), PGHS-2 (■□) mPGES (▲△) and cPGES (◆◇) (B).

Figure 3. PGES activities in splenic MØ isolated from BCG-immunized Balb/c mice. Groups of Balb/c mice received i.p. doses (0 [0.2 ml saline], 0.01 and 1 mg/dose) of HK-BCG (4 mice/group) on day 0. Day 14, spleens were harvested. Splenic MØ were isolated, pooled in each

group, and sonicated. PGES activities in cell lysates, converting from exogenous PGH_2 to PGE_2, were measured as described in the *Materials and Methods*. The levels of PGE_2 were measured by ELISA. Mean ± SD, n=3. In the absence of exogenous PGH_2, endogenous PGE_2 levels were no more than 0.87 ng/10 μg protein.

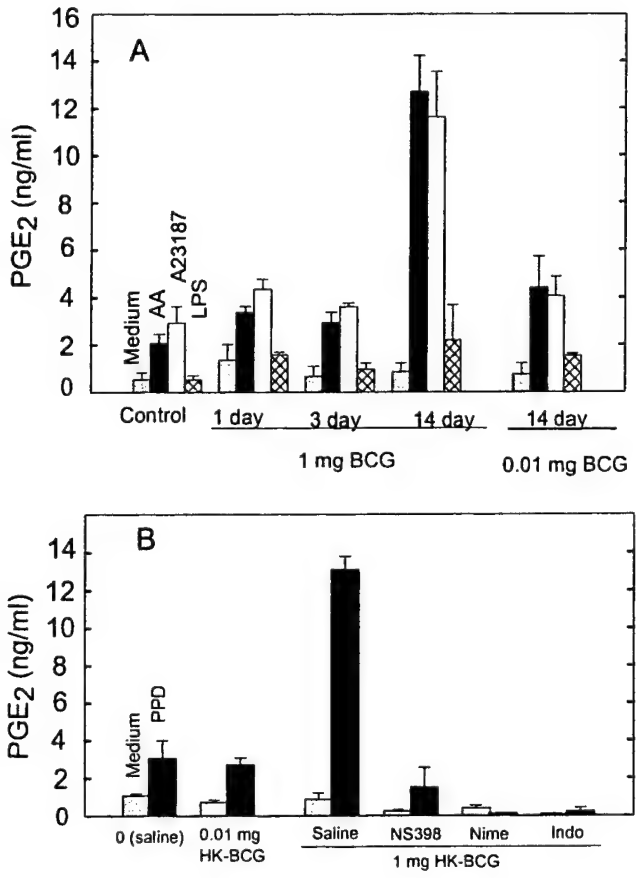
Figure 4. Total serum IgE levels and mycobacterial antigen-specific IgG1 and IgG2a levels. Groups of Balb/c mice received i.p. doses (0 [0.2 ml saline], 0.01 and 1 mg/dose) of HK-BCG (5 mice/group) on day 0. Day 14, sera were harvested. Total IgE levels in the sera were measured by a sandwich ELISA. The levels of PPD-specific IgG1/IgG2a and HSP-65-specific IgG1/IgG2a in sera were measured as described in Materials and Methods. The sera were diluted 1/100 and 1/20 with saline before they were assayed for antigen-specific IgG1 (open bars) and IgG2a (closed bars) levels, respectively. Values are mean plus standard deviations; n=5. *, **, and ***, $P < 0.05$, $P<0.01$, and $P<0.005$, respectively, compared to the saline control group. Each result represents a group of 5 mice from two experiments.

Figure 5. Recall responses detected by IFNγ and IL-10 levels in Balb/c mice. Spleen cells were isolated from BCG-immunized Balb/c mice receiving 0.01 mg or 1 mg of HK-BCG i.p. and stimulated *in vitro* with 50 μg/ml of PPD or 5 μg/ml of HSP65 for 4 days. In some cultures, nimesulide was added at 1 μM. The levels of IFNγ and IL-10 in the culture supernatants were measured by ELISA, as described in Materials and Methods. Values are means plus standard deviation from triplicate cultures. The data shown are representative of two independent experiments. *, **, and ***, $P < 0.05$, $P<0.01$, and $P<0.005$, respectively, compared to the saline control group. Each result represents a group of 5 mice from two experiments.

Figure 6. HK-BCG induces splenic PGE_2-MØ formation, the shift from Th1 to Th2 responses, and IgE formation in C57Bl/6 mice. Groups of C57Bl/6 mice received i.p. doses (0 [0.2 ml saline], 0.01 and 1 mg/dose) of HK-BCG (4 mice/group) on day 0. Day 14, spleens and blood were harvested. Splenic MØ were isolated, pooled in each group, incubated in serum-free RPMI 1640 medium (10^6 MØ/ml) containing calcium ionophore A23187 at 1 mM for 2 hrs. The levels of PGE_2 were measured by ELISA (Cayman) (A). Mean ± SD, n=3. Spleen cells were cultured in the presence of 50 μg/ml PPD for 4 days. The levels of IFNγ (closed bars) and IL-4 (opened bars) in the supernatants were measured by ELISA (PharMingen) (B). Mean ± SD, n=3. Serum total IgE levels were measured by ELISA (PharMingen) with mouse IgE κ isotype as a standard (C). Mean ± SD, n=4. *, **, and ***, $P < 0.05$, $P<0.01$, and $P<0.005$, respectively, compared to the saline control group.

Figure 7. PGHS inhibitors inhibit IL-4 production (Th2 cells) and decrease serum IgE levels, but enhance IFNγ production (Th1 cells). Groups of C57Bl/6 mice received i.p. 0.5 mg of HK-BCG (4 mice/group) on day 0 (closed bars). Controls received 0.2 ml of saline (opened bars). BCG-treated mice and their controls were treated with nimesulide (Nimes), niflumic acid (Niflum), or indomethacin (Indo) daily all at 50 mg/kg doses i.p. starting on day 1. Control mice received 0.5% ethanol in saline (0.2 ml). Day 14, spleens and sera were harvested and spleen cells were cultured in the presence of PPD at 10 mg/ml for 4 days. The levels of IL-4 (A) and IFNγ (B) in the supernatants and total IgE in sera (C) were measured by ELISA. Mean ± SD, n=3. *, $p<0.05$, **, $p<0.01$, compared to saline treatment. Each result represents a group of 5 mice from two experiments.

Figure 1.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PGE2 (ng/ml)
16
14
12
10
8
6
4
2
0
Medium
AA
A23187
LPS
Control
1 day
3 day
14 day
14 day
1 mg BCG
0.01 mg BCG
B
PGE2 (ng/ml)
14
12
10
8
6
4
2
0
Medium
PPD
0 (saline)
0.01 mg HK-BCG
Saline
NS398
Nime
Indo
1 mg HK-BCG

Figure 2.

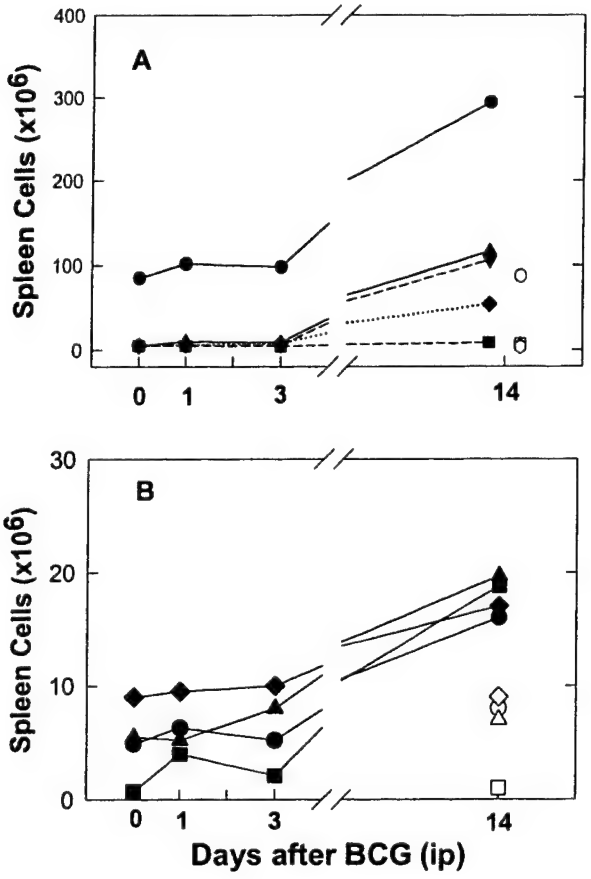

A
B
Spleen Cells (x10^6)
Spleen Cells (x10^6)
400
300
200
100
0
30
20
10
0
0 1 3 14
0 1 3 14
Days after BCG (ip)

Figure 3.

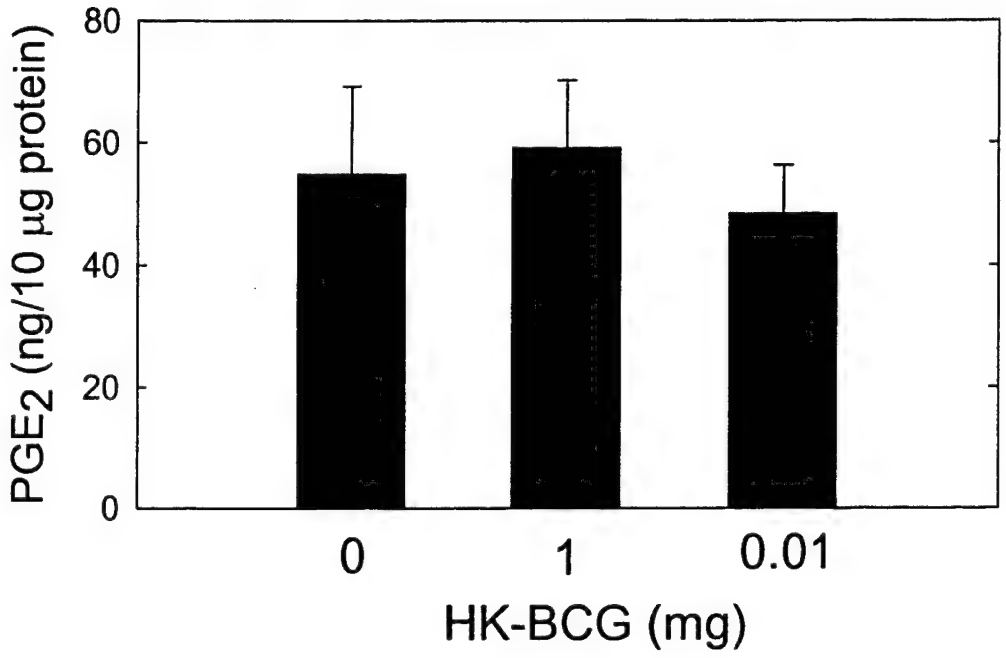


Figure 4.

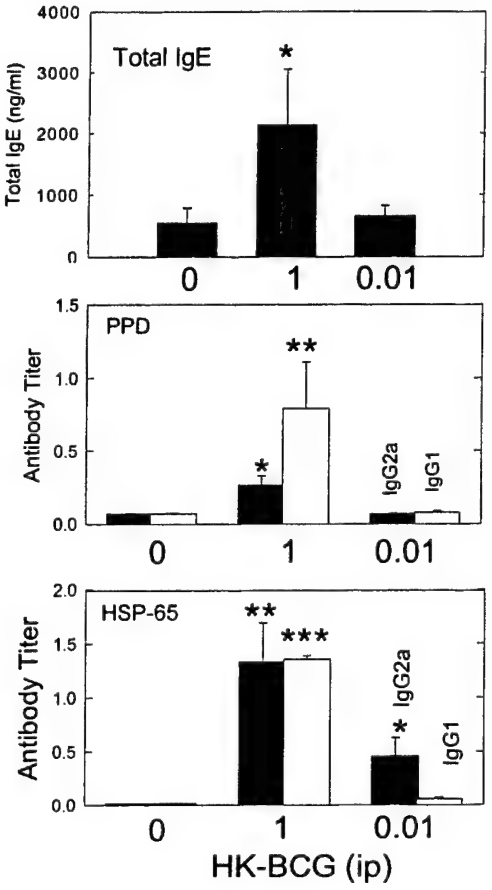

Total IgE
Total IgE (ng/ml)
4000
3000
2000
1000
0
*
0
1
0.01
PPD
Antibody Titer
1.5
1.0
0.5
0.0
**
*
IgG2a
IgG1
0
1
0.01
HSP-65
Antibody Titer
2.0
1.5
1.0
0.5
0.0
**
***
* IgG2a
IgG1
0
1
0.01
HK-BCG (ip)

Figure 5.

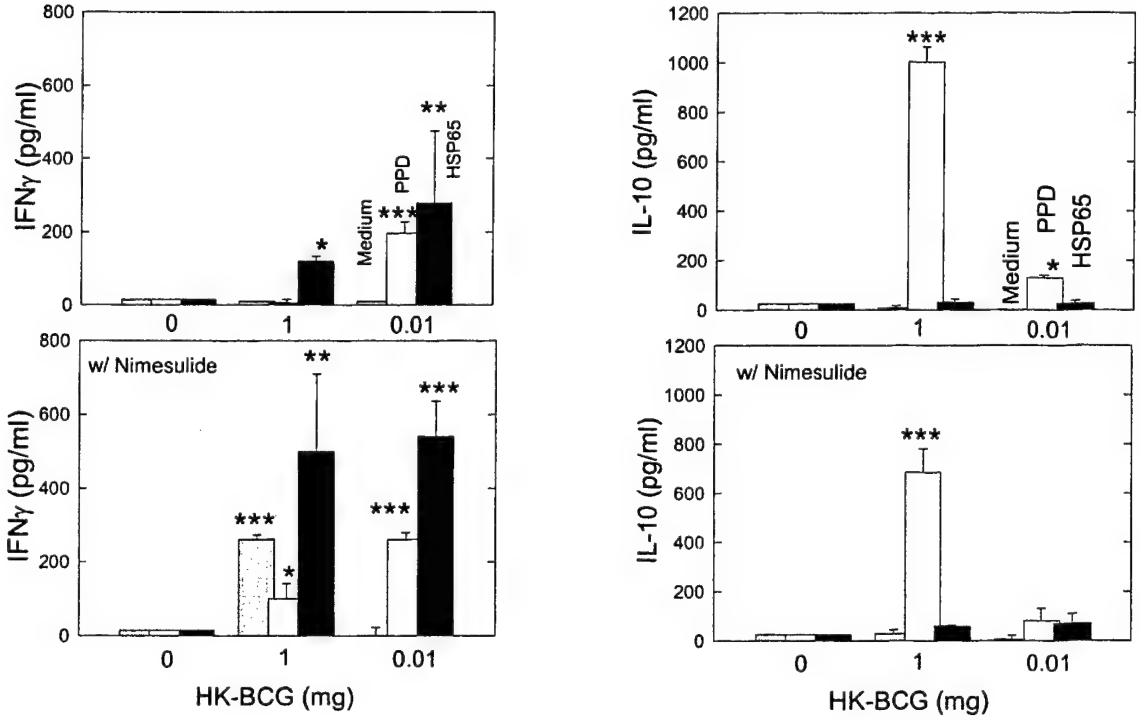

IFNγ (pg/ml)
800
600
400
200
0
Medium
PPD
HSP65
*
***
**
0
1
0.01
w/ Nimesulide
IL-10 (pg/ml)
1200
1000
800
600
400
200
HK-BCG (mg)

Figure 6.

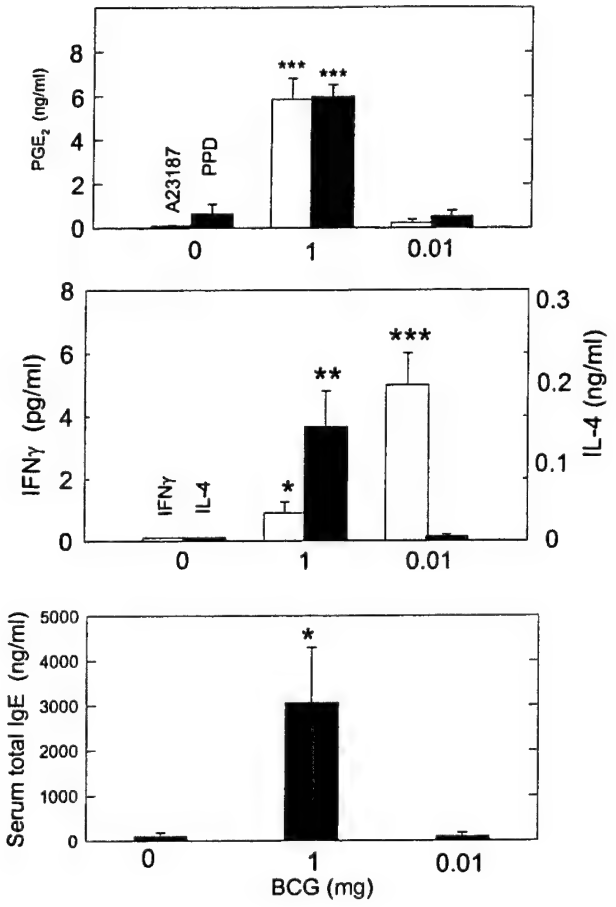
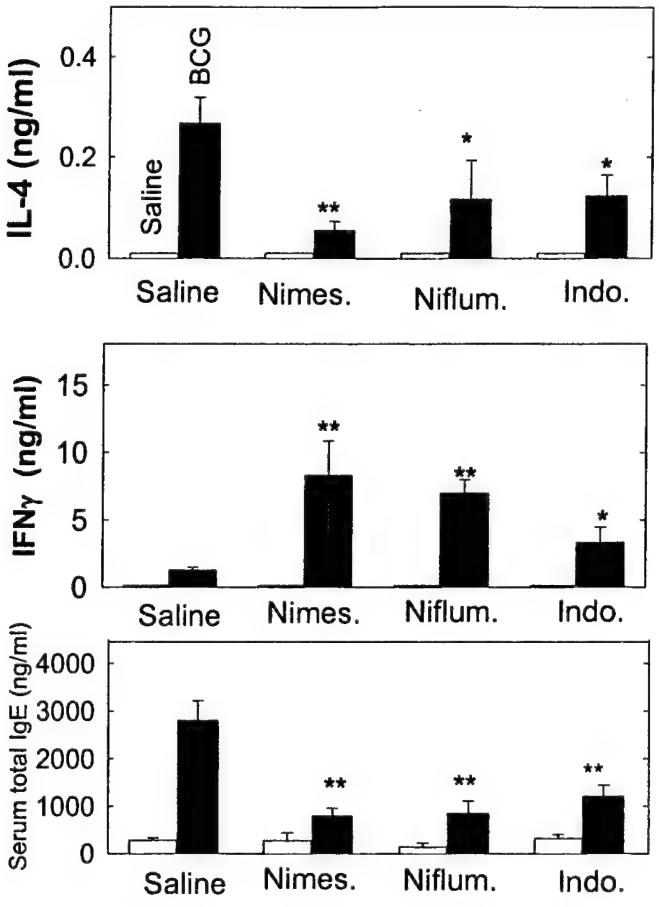

PGE2 (ng/ml)
A23187
PPD
***
***
0
1
0.01
IFNγ (pg/ml)
IL-4 (ng/ml)
IFNγ
IL-4
*
**
***
Serum total IgE (ng/ml)
BCG (mg)

Figure 7.


IL-4 (ng/ml)
0.4
0.2
0.0
Saline
BCG
**
*
*
Saline
Nimes.
Niflum.
Indo.
IFNγ (ng/ml)
15
10
5
0
**
**
*
Saline
Nimes.
Niflum.
Indo.
Serum total IgE (ng/ml)
4000
3000
2000
1000
0
**
**
**
Saline
Nimes.
Niflum.
Indo.

Differential effects of IL-10 on prostaglandin H synthase-2 expression and prostaglandin E_2 biosynthesis between spleen and bone marrow macrophages

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ABSTRACT

Comparisons of populations or compartments of mononuclear phagocytes (MØ) show considerable diversity of cellular function including PGE_2 biosynthesis. Treatments with bacterial components *in vitro* and *in vivo* can enhance PGE_2 biosynthesis indicating modulation is also important determinant of MØ diversity. IL-10 is proposed to inhibit the modulation of PGE_2 biosynthesis through down-regulating PGHS-2 expression in some MØ. To assess whether IL-10 regulates PGE_2 biosynthesis in splenic MØ and bone marrow MØ, these MØ were isolated from IL-10- deficient ($IL\text{-}10^{-/-}$), C57Bl/6 (wild type [WT] control) and Balb/c (comparison control) mice and treated *in vitro* with LPS and/or IFNγ. We evaluated the levels of PGE_2 release and PGHS-1 and 2 expressions. LPS-induced PGHS-2 expression in $IL\text{-}10^{-/-}$ spleen MØ was similar to that from splenic MØ isolated from Balb/c and C57Bl/6 mice. However, PGE_2 levels released by LPS-treated splenic MØ exhibited slight but significantly higher in $IL\text{-}10^{-/-}$ and Balb/c than those in WT mice. In the presence of LPS and IFNγ, PGHS-2 and PGE_2 production in both $IL\text{-}10^{-/-}$ and Balb/c mice was enhanced compared to stimulation with LPS alone or IFNγ alone. However, in C57Bl/6 WT mice, there was no increase in PGE_2 release in the presence of the combined mediators despite a synergistic increase in PGHS-2 expression. PGHS-1 protein levels were not affected by LPS stimulation in either WT or $IL\text{-}10^{-/-}$ mice. In sharp contrast, PGHS-2 and PGE_2 biosynthesis in bone marrow MØ were significantly enhanced in $IL\text{-}10^{-/-}$. Our results indicate various degrees of IL-10-mediated MØ PGE_2 biosynthesis and PGHS-2 expression that are compartment-dependent.

INTRODUCTION

Mononuclear phagocytes (MØ) are a major source of prostaglandin E_2 (PGE_2), an arachidonic acid (AA) metabolite, that regulates the immune response, hematopoiesis, inflammation, tissue injury and repair, and bone resorption. The regulation of these events may be closely related to the regulation of PGE_2 release by MØ (1). The effective *in vivo* expression of these functions, furthermore, may depend on the presence of an adequate number of MØ with appropriate functions in appropriate locations (2,3). We are interested in lymphoid tissues of spleen where PGE_2-releasing MØ (PGE_2-MØ) and immune lymphocytes interact in chronic inflammatory diseases including mycobacterial infections. PGE_2 inhibits the production of Th1 cytokines such as IL-2, IL-12 and IFNγ (4). In contrast, PGE_2, depending on stimulatory conditions, either has no effect or enhances production of Th2 cytokines, such as IL-4, IL-5 and IL-10 (4,5). Therefore, increases in splenic PGE_2-MØ may underlie the Th1-to-Th2 shift of immune responses that are major pathogenic events in chronic inflammatory diseases. More studies are needed to elucidate the mechanism of splenic PGE_2-MØ formation.

Unlike peritoneal MØ, bone marrow MØ, or monocytes, normal splenic MØ exhibit relatively low levels of PGE_2 biosynthesis (<1 ng of $PGE_2/10^6$ MØ/ml) (6). However, previous studies (7) have shown that an *in vivo* response to chronic inflammatory conditions including mycobacterial infections is manifested by the emergence of splenic MØ with the capacity to form large amounts of PGE_2 (>10 ng/ml). The mechanisms for splenic PGE_2-MØ formation appear to be complex. Our studies have shown that their formation is dependent on radiosensitive bone

marrow cells, which may supply precursors of splenic PGE_2-MØ (2). It is likely that the precursors migrate and localize to the spleen where mature forms of PGE_2-MØ are established (6). Alternatively, an inflammatory cytokine "milieu" may directly up-modulate PGE_2 biosynthesis of splenic MØ (8,9).

PGE_2-MØ metabolize endogenous arachidonic acid (AA) to PGE_2 through cyclooxygenase (PGHS, EC 1.4.99.1), a rate-limiting enzyme for prostaglandins, thromboxane and prostacyclin. Two major isoforms of PGH synthase exist: PGHS-1, a constitutive form; and PGHS-2, an inducible form that is rapidly up-regulated in response to LPS and cytokines (10-12). The formation of splenic PGE_2-MØ must, therefore, be dependent on the levels of PGHS-2 induced (8).

Cytokines, which represent pro- and/or anti- inflammatory conditions, modulate PGE_2 synthesis by MØ cell lines (13) and MØ freshly isolated from the peritonea, and lungs or derived from monocytes (8,11,12,14). TNFα (15), IL-1α (15,16), and IFNγ (17,18) have been demonstrated to induce PGHS-2 expression, whereas IL-4 (19), IL-13 (20,21), IL-10 (22), and TGF-β (23) can inhibit PGHS-2 induction. The question addressed in these studies is whether the level of PGHS-2 expression is regulated by selected inflammatory conditions directly linked to PGE_2 biosynthesis in splenic MØ.

IL-10 is a MØ deactivator, blocking LPS-induced synthesis of TNFα, IL-1β, IL-6, IL-8, IL-12 and GM-CSF by human monocytes (24), mouse peritoneal MØ (25) and mouse splenic MØ

(26). It has recently been reported that IL-10 can inhibit PGHS-2 induction *in vitro* in human monocytes and neutrophils (19,22). Recently, Berg et al (8) demonstrated that LPS induces normal mouse spleen MØ to express PGHS-2 and PGE_2 biosynthesis that are down-regulated by endogenous IL-10. Our preliminary studies, however, showed that IL-10 down-regulated PGE_2 synthesis with less likely down-regulating PGHS-2 expression. Furthermore, IL-10 showed remarkably greater inhibition of PGE_2/PGHS-2 synthesis in bone marrow MØ.

MATERIALS AND METHODS

Animals. Healthy 8- to 12-wk-old IL-$10^{-/-}$ female mice on C57Bl/6 background were used for this study. Wild-type C57Bl/6 and Balb/c female mice were obtained from Jackson Lab (Bar Harbor, ME). All mice were maintained in microisolator cages under specific pathogen-free conditions at the animal care facility at East Carolina University.

Reagents. LPS from *Escherichia coli* (serotype 0111:B4, phenol extraction, L-2630) and mouse recombinant IFNγ (I-4777) were obtained from Sigma, St. Louis, MO and reconstituted in pyrogen-free saline. A23187 (Sigma) was dissolved in DMSO at 1 mg/ml. AA (Sigma) was dissolved in 100% ethanol at 1 mg/ml. Rabbit polyclonal anti-murine PGHS-1 and rabbit polyclonal anti-PGHS-2 were obtained from Cayman Chemicals (Ann Arbor, MI).

MØ preparations from spleen and bone marrow. Spleens in each group of mice (at least 5 mice per group) were isolated and pooled. Excised spleens were minced with scissors and digested with collagenase D (C-2139, Sigma) at 50 U/ml in RPMI 1640 plus 10% FBS at 37°C for 60 min in and filtered through a 100-μm mesh. After washing digested cells with RPMI 1640 in the presence of DNase at 100 μg/ml (DN-25, Sigma), cells were suspended in RPMI 1640 plus 10% FBS. Mouse bone marrow cells were isolated by flushing the marrow cavities of the femurs with ice-cold RPMI 1640 medium and gently refluxing the expelled cell plug with a Pasteur pipette to form a single cells suspension. To enrich MØ fraction (2,6), spleen cell and bone marrow cell suspensions were layered over top of a discontinuous Percoll gradient (35/60%,

Sigma). Following centrifugation (800xg for 30 min at 22°C), cells in the layer between 35% and 60% Percoll were collected, washed and suspended in RPMI 1640 medium plus 10% FBS. These cells were plated at approximately 3 – 5 x 10^6 cells/ml per 35 mm culture dish (Falcon, Oxnard, CA) and incubated at 37°C in 5% CO_2 in air. After 2 hr incubation, the cells were washed with Ca^{2+} and Mg^{2+} -free 0.15 M phosphate-buffered saline (PBS) for removal of the nonadherent cells. Following placing the dish on ice for 30 minutes, the adherent cells were harvested by scraping and washed twice with serum-free RPMI 1640. Viability was >90% (trypan blue exclusion). Adherent spleen cells were >85% MØ; adherent marrow cells were >92%, both estimated with phagocytosis of IgG-opsonized sheep red cells (2) and/or cytometrically following staining with anti-Mac-1 (27).

Cell culture protocols. Splenic MØ and bone marrow MØ were isolated above and cultured at 5 x 10^6 cells/ml in RPMI 1640 supplemented with 5 % FBS, penicillin (100 U/ml), streptomycin (100 U/ml) and amphotericin B (2.5 μg/ml) in 12 x 75 mm culture tubes (0.5 ml/tube, Costar, Corning, NY). Cells were incubated in medium alone or medium supplemented with LPS at 10 μg/ml, IFNγ (10 ng/ml) or LPS plus IFNγ mixture. After 24 hrs, the cells were washed with cold saline and treated with a lysis buffer as described below. To elicit PGE_2 release, the cultured cells were washed, suspended in serum-free RPMI 1640, and incubated with agonists (A23187, AA, LPS) at 37°C. Supernatants from triplicate cultures were harvested after 2 hr and stored at -70°C before analysis for PGE_2.

Quantification of PGE_2 and TxB_2. PGE_2 and TxB_2 levels in tissue culture supernatants were determined using enzyme immunoassay kits (Cayman Chemicals) according to the manufacturer's instructions.

Western blotting. Splenic MØ and bone marrow MØ were cultured as described above, harvested and washed 3 times with cold saline. Protein was isolated from washed cells by resuspending in lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 100 μg/ml PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM diethyldithiocarbamic acid, 1% Nonidet P-40, and 1% sodium deoxycholate). Debris was eliminated by centrifugation (5 min, 1000 x *g*). Protein concentration was measured using a commercial reagent based on bicinchoninic acid staining (Pierce, Rockford, IL) using BSA as standard. Equal amounts of cellular protein were loaded onto SDS-polyacrylamide gel and separated by electrophoresis (200 V for 45 min). Proteins were then transferred to PVDF membrane (100 V for 1 h), and the membrane was blocked with 5% nonfat dry milk. The membrane was then incubated with antibody (anti-PGHS-1, 1:1000; anti-PGHS-2, 1:1000) overnight at 4°C. Following incubation with peroxidase conjugated donkey anti rabbit IgG (Jackson ImmunoResearch, West Glove, PA), proteins were detected by chemiluminescence (ECL, Amersham, Piscataway, NJ) per the manufacturer's instructions.

Statistics. Data from this project were analyzed by one way analysis of variance. For cell culture studies, tissues isolated from at least 5 mice were pooled unless indicated; these cells

were cultured in at least triplicate in each group. *P* value of less than 0.05 is considered statistically significant.

RESULTS

PGE_2 release by splenic MØ isolated from IL-$10^{-/-}$, C57Bl/6 (WT), and Balb/c mice. Normal murine splenic MØ release PGE_2 at minimum levels compared to bone marrow MØ (3,6). To determine whether endogenous IL-10 regulates PGE_2 released by splenic MØ in response to the proinflammatory stimuli LPS and/or IFNγ, splenic MØ were isolated from C57Bl/6 (WT), and IL-$10^{-/-}$ mice. Recently, Kuroda and Yamashita (9) found differential release of PGE_2 by splenic MØ isolated from C57Bl/6 and Balb/c mice when stimulated by LPS *in vitro*. We, therefore, employed splenic MØ isolated from Balb/c mice as comparison controls. The MØ were primed with LPS (10 μg/ml), IFNγ (10 ng/ml), LPS combined with IFNγ, or medium alone for 24 hr, followed by the elicitation of PGE_2 release with 1 μM calcium ionophore A23187 for 2 hrs. As shown in Figure 1, PGE_2 release was slightly but significantly enhanced when splenic MØ were primed with LPS in Balb/c and IL-$10^{-/-}$ mice. Although IFNγ did not prime PGE_2 biosynthesis by itself, but it showed a synergistic effect when mixed with LPS. Such priming effects of LPS and IFNγ were not observed in splenic MØ from C57Bl/6 mice (Figure 1).

PGE_2 release by bone marrow MØ isolated from IL-$10^{-/-}$, C57Bl/6 (WT), and Balb/c mice. When treated with 1 μM A23187, bone marrow MØ from Balb/c, C57Bl/6 or IL-$10^{-/-}$ mice released 10-fold more PGE_2 than normal splenic MØ (Figure 1). PGE_2 release was not increased when bone marrow MØ from Balb/c and C57Bl/6 mice were primed with LPS. LPS plus IFNγ significantly enhanced PGE_2 release in Balb/c but not C57Bl/6 mice. In sharp contrast, bone marrow MØ from IL-$10^{-/-}$ mice showed 10-fold increase in PGE_2 release in response to LPS

alone, without further enhancement by the mixture of IFNγ and LPS (Figure 1). Elicitation with AA at 1 μg/ml also showed similar profiles of PGE_2 release but at significantly lower amounts among the treatment groups than those elicited by A23187 (Table 1). Table 1 also shows PGE_2 levels that were elicited by LPS at 1 μg/ml. However, the elicitation by LPS did not significantly enhance PGE_2 production compared to that by buffer alone in any primed groups (data not shown).

There was no increase of thromboxane (TxB_2) release by splenic MØ isolated from C57Bl/6 and Balb/c mice that were pre-treated with LPS and/or IFNγ. Thromboxane production was not enhanced in IL-$10^{-/-}$ mice (Figure 2). Furthermore, as shown in Figure 2, bone marrow MØ from C57Bl/6 and Balb/c mice released TxB_2 at significantly higher levels compared to those from splenic MØ. However, priming with LPS and IFNγ did not result in increases in TxB_2 release, but rather the suppression of thromboxane production (Figure 2). Our results clearly demonstrated that endogenous IL-10 had no significant effect on the thromboxane production by splenic or bone marrow MØ (Figure 2).

Effect of endogenous IL-10 on protein expression of PGHS-1 and PGHS-2. To determine whether endogenous IL-10 alters the level of protein expression of PGHS-1 and PGHS-2, Western blot analyses employing specific antibodies against PGHS-1 and PGHS-2 was performed. Untreated splenic MØ isolated from Balb/c, C57Bl/6 and IL-$10^{-/-}$ mice showed PGHS-1 expression and the levels were relatively stable following the treatments with IFNγ and/or LPS (Figure 3). On the other hand, levels of PGHS-2 protein in splenic MØ isolated from Balb/c, C57Bl/6 and IL-$10^{-/-}$ mice were undetectable. Stimulation of splenic MØ with LPS resulted in marked increases in PGHS-2 protein at comparable levels among the three strains of

mice. The mixture of LPS and IFNγ further enhanced PGHS-2 levels in all strains, although PGHS-2 proteins were slightly more increased in Balb/c than in C57Bl/6 and IL-$10^{-/-}$ (Figure 3). Our results demonstrated significant enhancement of PGHS-2 protein levels in the absence of IL-10.

Figure 4 shows that bone marrow MØ from Balb/c and C57Bl/6 mice showed increases in PGHS-2 levels in response to LPS. These levels were further enhanced by LPS combined with IFNγ (Figure 4). It is of particular distinction that PGHS-2 levels that were induced by LPS or LPS + IFNγ were significantly enhanced in IL-$10^{-/-}$ mice compared to C57Bl/6 and Balb/c mice (Figure 4). Bone marrow MØ PGHS-1 expression was unchanged among the treatment groups as well as among the three mouse strains (Figure 4).

DISCUSSION

Regulation of PGE_2 Production. PGE_2 is a key mediator regulating inflammatory responses (28-33). Under chronic inflammatory conditions, increases in splenic PGE_2-MØ potentially induce Th1-to-Th2 shifts of the immune response that is a major pathogenic event in mycobacterial infections and other chronic inflammatory diseases. The mechanisms regulating PGE_2 synthesis by MØ at local sites of inflammation appear to be complex. In addition, heterogeneity of PGE_2 biosynthesis among MØ populations has been well documented (3,34). Normal splenic MØ express relatively low levels of PGE_2 (<1 ng of $PGE_2/10^6$ MØ/ml). However, 7 to 14 days after mice are challenged with mycobacteria, splenic MØ show a 10-fold increase in PGE_2 release (>10 ng/ml) (2,3,6). These *in vitro* studies were undertaken to examine the role of IL-10 in regulating the PGE_2 response by MØ in the presence of specific inflammatory mediators.

LPS Priming of Splenic MØ. In vitro priming of normal splenic MØ with LPS enhances PGE_2 synthesis, but only to less than 2 ng/ml (8,9). We have determined the contribution of endogenous IL-10 to PGE_2 biosynthesis and the expression of PGHS-2 in splenic MØ. Our studies demonstrate that LPS induces PGHS-2 protein expression by IL-$10^{-/-}$ splenic MØ with a profile similar to that seen in splenic MØ from C57Bl/6 (WT) or Balb/c mice. However, comparing IL-$10^{-/-}$ mice to their WT controls, LPS-primed splenic IL-$10^{-/-}$ MØ produce slightly but significantly higher levels of PGE_2. Nevertheless, these PGE_2 levels are relatively low compared to levels produced by bone marrow MØ (Figure 1). Interestingly, the levels of PGE_2 synthesis in the presence or absence of IL-10 do not directly correlate with PGHS-1 or PGHS-2 levels (Figure 3).

Contribution of IFNγ. IFNγ in synergy with LPS is reported to induce PGHS-2 expression in peritoneal MØ (35) through the activation of NF-κB (36). Our results with splenic MØ indicate that in the presence of this combination of mediators, PGHS-2 and PGE_2 production in both IL-$10^{-/-}$ and Balb/c mice is enhanced compared to stimulation with LPS alone or IFNγ alone. However, in C57Bl/6 WT mice, there was no increase in PGE_2 release in the presence of the combined mediators despite a synergistic increase in PGHS-2 expression. Comparing IL-$10^{-/-}$ mice to the C57Bl/6 controls IL-10 at levels seen in splenic MØ under chronic inflammatory conditions appears to suppress PGE_2 synthesis, but, in the presence of IFNγ, may enhance PGHS-2 expression (Figures 1 & 3). Our *in vitro* studies also suggest that IL-10 is less likely to regulate thromboxane production (Figure 2). In addition the results for Balb/c and C57Bl/6 mice indicate a genetically determined difference in the murine inflammatory response.

Dissociation of PGE_2 Production from Induction of PGHS-2. Kuroda and Yamashita (9) also demonstrated dissociation between PGHS-2 expression and PGE_2 biosynthesis in the *in vitro* activation of splenic MØ from Balb/c and C57Bl/6 mice. In our previous studies (2,3,6,37) of mice including IL-$10^{-/-}$ and WT mice immunized with BCG or *C. parvum*, establishment of high PGE_2-releasing splenic MØ required 7 – 14 days (>10 ng of $PGE_2/10^6$ MØ). However, splenic MØ isolated one day after immunization express PGHS-2 protein without increased PGE_2 production (<1 ng/ml) (unpublished data). This *in vivo* observation would be analogous to the *in vitro* induction of PGHS-2 by LPS and/or IFNγ. Since the PGHS-1 level is relatively unchanged either *in vitro* or *in vivo*, another enzyme such as PGE synthase may be involved in the regulation of PGE_2 biosynthesis in an IL-10-dependent manner. One cannot rule out the

contribution of PGHS-3, a PGHS-1 variant that is recently identified (38). However, probably phospholipase A_2 may not be involved because of any effect on thromboxane production.

PGE_2 Synthesis by Bone Marrow MØ. In bone marrow MØ of C57Bl/6 WT mice, we found that, as for splenic MØ, LPS in synergy with IFNγ induced PGHS-2 but not PGE_2 synthesis. Unlike splenic MØ, LPS-stimulated marrow IL-$10^{-/-}$ MØ showed marked increases in both PGHS-2 and PGE_2 biosynthesis compared with C57Bl/6 (WT). For bone marrow MØ in contrast to splenic MØ, there was a less pronounced difference in PGE_2 production in the presence of LPS between C57Bl/6 WT and Balb/c mice. Previous studies of PGE_2 production in response to LPS, inflammatory cytokines, and/or mitogens concluded that PGE_2 production is significantly associated with an increase in PGHS-2 protein levels (12). Our findings for bone marrow MØ from IL-$10^{-/-}$ mice are consistent with this observation, but for the wild type mice the correlation appears to be less direct. In contrast, IL-10 did not appear to exert a regulatory effect on PGHS-1 protein expression, similar to previous reports indicating that PGHS-1 expression is generally constitutive rather than inducible (8).

Role of IL-10. There are multiple possible mechanisms for IL-10 regulation of PGHS-2 synthesis (8). It is established that LPS and IL-1β enhance PGHS-2 mRNA stability (39), whereas exogenous IL-10 *in vitro* accelerates the degradation of PGHS-2 mRNA in human monocytes (22). Regardless of how endogenous IL-10 regulates PGE_2 synthesis, in the presence of IL-10, C57Bl/6 splenic MØ activated by LPS and IFNγ produce a maximum of 2 ng/ml of PGE_2/5 x 10^6 MØ. Additional factors must contribute to the enhanced PGE_2 formation by splenic PGE_2-MØ isolated from mice 7 – 14 days after BCG immunization (6,37). These results

are consistent with our previous hypothesis that splenic PGE_2-MØ are derived from radiosensitive bone marrow cells (2,6). In conclusion these *in vitro* studies have shown that for the murine C57Bl/6 strain, IL-10 contributes to the up regulation of PGHS-2 synthesis in bone marrow, but not in splenic macrophages and that endogenous IL-10 significantly suppresses PGE_2 production in both splenic and marrow MØ previously primed with LPS. Thus, IL-10 may exert both pro-inflammatory and anti-inflammatory effects on MØ. The results further indicate that PGE_2 production is not solely the consequence of the PGHS enzyme level and that additional factors must regulate PGE_2 production.

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REFEREMCES

1. Dubois, R. N., S. B. Abramson, L. Crofford, R. A. Gupta, L. S. Simon, L. B. Van De Putte, P. E. Lipsky. 1998. Cyclooxygenase in biology and disease. *FASEB J. 12:1063.*

2. Shibata, Y., A. Volkman. 1985. The effect of bone marrow depletion on prostaglandin E producing suppressor macrophages in mouse spleen. *J. Immunol. 135, 3897.*

3. Shibata, Y., A. P. Bautista, S. N. Pennington, J.L. Humes, A. Volkman. 1987. Eicosanoid production by peritoneal and splenic macrophages in mice depleted of bone marrow by ^{89}Sr. *Am. J. Pathol., 127, 75.*

4. Rocca, B., L. M. Spain, E. Pure, R. Langenbach, C. Patrono, G. A. Fitzgerald. 1999. Distinct roles of prostaglandin H synthase 1 and 2 in T-cell development. *J. Clin Invest. 103:1469.*

5. Fitzgerald, T. J. 1992. The Th1/Th2 switch in syphilitic infection: is it detrimental? *Infect. Immun. 60:3475.*

6. Shibata, Y. 1989. Restoration of prostaglandin-producing splenic macrophages in ^{89}Sr-treated mice with bone marrow from *Corynebacterium parvum* primed donors. *Regional Immunol., 2, 169.*

7. Druker, B. J., H. T .Wepsic. 1983. BCG-induced macrophages as suppressor cells. *Cancer Investig. 1:151.*

8. Berg, D. J., J. Zhang, D. M. Lauricella, S. A. Moore. 2001. IL-10 is a central regulator of cyclooxygenase-2 expression and prostaglandin production. *J. Immunol 166:2674.*

9. Kuroda, E., U. Yamashita. 2003. Mechanisms of enhanced macrophage-mediated prostaglandin E2 production and its suppressive role in Th1 activation in Th2-dominant Balb/c mice. *J. Immunol. 170:757.*

10. Smith, W. L., R. M. Garavito, D. L. DeWitt. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem. 271:33157.*

11. Endo, T., F. Ogushi, S. Sone. 1996. LPS-dependent cyclooxygenase-2 induction in human monocytes is down-regulated by IL-13, but not by IFN-γ. *J. Immunol. 156:2240.*

12. Lee, S. H., E. Soyoola, P. Chanmugam, S. Hart, H. Zhong, S. Liou, D. Simmons, D. Hwang. 1992. Selective expression of mitogen-inducible cyclooxygenase in macrophages with lipopolysaccharide. *J. Biol. Chem. 267:25934.*

13. Giroux, M., A. Descoteaux. 2000. Cyclooxygenase-2 expression in macrophages: modulation by protein kinase C-alpha. *J. Immunol. 165:3985.*

14. O'Sullivan, M. G., E. M. Huggins Jr., E. A. Meade, D. L. DeWitt, C. E. McCall. 1992. Lipopolysaccharide induces prostaglandin H synthase-2 in alveolar macrophages. *Biochem. Biophys. Res. Commun. 187:1123.*

15. Vlahos, R., A. G. Stewart. 1999. Interleukin-1α and tumour necrosis factor-α modulate airway smooth muscle DNA synthesis by induction of cyclo-oxygenase-2: inhibition by dexamethasone and fluticasone propionate. *Br. J. Pharmacol. 126:1315.*

16. Huang, Z. F., J. B. Massey, D. P. Via. 2000. Differential regulation of cyclooxygenase-2 (COX-2) mRNA stability by interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) in human in vitro differentiated macrophages. *Biochem. Pharmacol. 59:187.*

17. Barrios-Rodiles, M., K. Chadee. 1998. Novel regulation of cyclooxygenase-2 expression and prostaglandin E_2 production by IFN-γ in human macrophages. *J. Immunol. 161:2441.*

18. Matsuura, H., M. Sakaue, K. Subbaramaiah, H. Kamitani, T. E. Eling, A. J. Dannenberg, T. Tanabe, H. Inoue, J. Arata, A. M. Jetten. 1999. Regulation of cyclooxygenase-2 by interferon γ and transforming growth factor αin normal human epidermal keratinocytes and squamous carcinoma cells: role of mitogen-activated protein kinases. *J. Biol. Chem. 274:29138.*

19. Niiro, H., T. Otsuka, K. Izuhara, K. Yamaoka, K. Ohshima, T. Tanabe, S. Hara, Y. Nemoto, Y. Tanaka, H. Nakashima, Y. Niho. 1997. Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood 89:1621.*

20. Diaz-Cazorla, M., D. Perez-Sala, J. Ros, W. Jimenez, M. Fresno, S. Lamas. 1999. Regulation of cyclooxygenase-2 expression in human mesangial cells: transcriptional inhibition by IL-13. *Eur. J. Biochem. 260:268.*

21. Alaaeddine, N., J. A. Di Battista, J. P. Pelletier, K. Kiansa, J. M. Cloutier, J. Martel-Pelletier. 1999. Inhibition of tumor necrosis factor α-induced prostaglandin E_2 production by the antiinflammatory cytokines interleukin-4, interleukin-10, and interleukin-13 in osteoarthritic synovial fibroblasts: distinct targeting in the signaling pathways. *Arthritis Rheum. 42:710.*

22. Niiro, H., T. Otsuka, T. Tanabe, S. Hara, S. Kuga, Y. Nemoto, Y. Tanaka, H. Nakashima, S. Kitajima, M. Abe. 1995. Inhibition by interleukin-10 of inducible cyclooxygenase expression in lipopolysaccharide-stimulated monocytes: its underlying mechanism in comparison with interleukin-4. *Blood 85:3736.*

23. Reddy, S. T., R. S. Gilbert, W. Xie, S. Luner, H. R. Herschman. 1994. TGF-β 1 inhibits both endotoxin-induced prostaglandin synthesis and expression of the TIS10/prostaglandin synthase 2 gene in murine macrophages. *J. Leukocyte Biol. 55:192.*

24. de Waal-Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, J. E. de Vries. 1991. Interleukin-10 inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med. 174:1209.*

25. Fiorentino, D. F., A. Zlotnik, T. R. Mosmann, M. Howard, A. O'Garra. 1991. IL-10 inhibits cytokine production by activated macrophages. *J. Immunol. 147:3815.*

26. Shibata, Y., L. A. Foster, M. Kurimoto, H. Okamura, R. M. Nakamura, K. Kawajiri, J. P. Justice, M. R. Van Scott, Q. N. Myrvik, W. J. Metzger. 1998. Immunoregulatory roles of IL-10 in innate immunity --- IL-10 inhibits macrophage production of IFN-gamma-inducing factors, but enhances NK cell production of IFN-gamma. *J. Immunol. 161:4283.*

27. Shibata, Y., D. R. Bjorkman, M. Schmidt, Y. Oghiso, A. Volkman. 1994. Macrophage colony-stimulating factor-induced bone marrow macrophages do not synthesize or release prostaglandin E2. *Blood 83:3316.*

34. Humes, J. L., R. J. Bonney, L. Pelus, M. E. Dahlgren, S. J. Sadowski, F. A. Kuehl Jr., P. Davies. 1977. Macrophages synthesis and release prostaglandins in response to inflammatory stimuli. *Nature 269:149.*

35. Blanco J. C . C., C. Contrusi, C. A. Salkowski, D. L. Dewitt, K. Ozato, S. N. Vogel. 2000. Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon γ-dependent cyclooxygenase 2 expression. *J. Exp. Med. 191:2131.*

36. Ohmori, Y., T. A. Hamilton. 2001. Requirement for STAT1 in LPS-induced gene expression in macrophages. *J. Leukoc. Biol. 69:598.*

39. Barrios-Rodiles, M., G. Tiraloche, K. Chadee. 1999. Lipopolysaccharide modulates cyclooxygenase-2 transcriptionally and posttranscriptionally in human macrophages independently from endogenous IL-1β and TNF-α. *J. Immunol. 163:963.*

28. Portanova, J. P., Y. Zhang, G. D. Anderson, S. D. Hauser, J. L. Masferrer, K. Seibert, S. A. Gregory, P. C. Isakson. 1996. Selective neutralization of prostaglandin E_2 blocks inflammation, hyperalgesia, and interleukin 6 production in vivo. *J. Exp. Med. 184:883.*

29. van der Pouw Kraan, T. C., L. C. Boeije, R. J. Smeenk, J. Wijdenes, L. A. Aarden. 1995. Prostaglandin-E_2 is a potent inhibitor of human interleukin 12 production. *J. Exp. Med. 181:775.*

30. Wu, C. Y., K. Wang, J. F. McDyer, R. A. Seder. 1998. Prostaglandin E_2 and dexamethasone inhibit IL-12 receptor expression and IL-12 responsiveness. *J. Immunol. 161:2723.*

31. Kalinski, P., C. M. Hilkens, A. Snijders, F. G. Snijdewint, M. L. Kapsenberg. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E_2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol. 159:28.*

32. Ferreri, N. R., T. Sarr, P. W. Askenase, N. H. Ruddle. 1992. Molecular regulation of tumor necrosis factor-α and lymphotoxin production in T cells. Inhibition by prostaglandin E_2. *J. Biol. Chem. 267:9443.*

33. Takano, M., H. Nishimura, Y. Kimura, J. Washizu, Y. Mokuno, Y. Nimura, Y. Yoshikai. 1998. Prostaglandin E_2 protects against liver injury after *Escherichia coli* infection but hampers the resolution of the infection in mice. *J. Immunol. 161:3019.*

37. Shibata Y., I. Honda, J. P. Justice, M. R. Van Scott, R. M. Nakamura, Q. N. Myrvik. 2001. Th1 adjuvant N-acetyl-D-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. *Infect. Immun. 69:6123.*

38. Chandrasekharan, N. V., H. Dai, K. L.Roos, N. K. Evanson, J. Tomsik, T. S. Elton, D. L. Simmons. 2002. COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci. U S A. 99:13926.*

Footnote

Abbreviations used in this paper: PGHS, prostaglandin G/H synthase; WT, wild-type; KO, homozygous genetic deficiency; MØ, macrophages; CMI, cell-mediated immunity; BCG, Bacillus Calmette-Guérin; PGE_2, prostaglandin E_2; HK, heat killed;

Table I. PGE_2 release by primed bone marrow MØ that were treated with A23187, AA or LPS

Priming (24 hr)	Elicitation (2 hr)		
	A23187 (1 μM)	AA (1 μg/ml)	LPS (1 μg/ml)
		(ng PGE_2/5 x 10^6 MØ)	
IL10-KO Bone marrow MØ			
Medium	14.5 ± 1.5	9.8 ± 2.6	4.0 ± 0.3
IFNγ	19.4 ± 2.8	11.0 ± 3.1	5.9 ± 0.8
LPS	146.7 ± 12.1	40.9 ± 6.4	16.5 ± 4.0
IFNγ + LPS	131.3 ± 16.5	37.0 ± 5.2	15.5 ± 2.8
C57Bl/6 Bone marrow MØ			
Medium	21.3 ± 5.8	19.1 ± 1.6	4.2 ± 0.1
IFNγ	16.0 ± 1.7	10.4 ± 0.9	3.8 ± 1.2
LPS	23.1 ± 1.1	16.5 ± 0.5	7.3 ± 0.7
IFNγ + LPS	29.9 ± 3.5	19.1 ± 1.2	7.7 ± 0.5

Bone marrow MØ (5 x 10^6 MØ/ml) isolated from IL-10-KO and WT (C57Bl/6) mice were primed with LPS (10 μg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ, or medium alone for 24 hr. These MØ were elicited by 1 μM A23187, 1 μg/ml AA, or 1 μg/ml LPS for 2 hr. PGE_2 levels in the supernatants were measured by ELISA. Mean ± SD, n=3. A part of values of PGE_2 are identical to those shown in Figure 1B. The values of PGE_2 at saline were no statistically significant compared to those of LPS (data not shown).

FIGURE LEGENDS

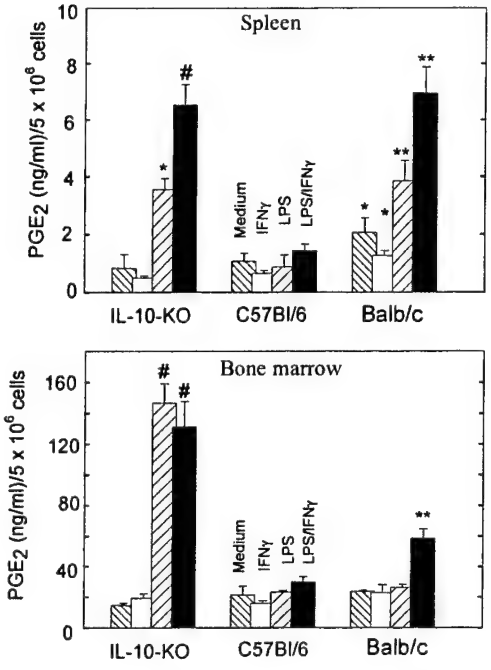
Figure 1. The effects of IL-10 on PGE_2 biosynthesis in splenic and bone marrow MØ. Splenic and bone marrow MØ (5×10^6 MØ/ml) isolated from IL-$10^{-/-}$, WT (C57Bl/6), and Balb/c mice were primed with LPS (10 μg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ, or medium alone for 24 hr. These MØ were elicited by 1 μM A23187 for 2 hrs and PGE_2 levels in the supernatants were measured by ELISA. Mean ± SD, n=3. $*p<0.05$, $**p<0.01$, $^{\#}p<0.001$ compared to those of C57Bl/6 (WT) mice. Results represent two separate experiments.

Figure 2. The effects of IL-10 on TxB_2 release by splenic and bone marrow MØ. Splenic and bone marrow MØ (5×10^6 MØ/ml) isolated from IL-$10^{-/-}$ and Balb/c mice were primed with LPS (10 μg/ml), IFNγ (10 ng/ml), LPS mixed with IFNγ, or medium alone for 24 hr. These MØ were elicited by 1 μM A23187 for 2 hrs and TxB_2 levels in the supernatants were measured by ELISA. Mean ± SD, n=3. $*p<0.05$, $**p<0.01$, $^{\#}p<0.001$ compared to those of C57Bl/6 (WT) mice. Results represent two separate experiments.

Figure 3. PGHS-1 and PGHS-2 levels in splenic MØ that were isolated from IL-$10^{-/-}$, WT (C57Bl/6), and Balb/c mice and primed with LPS and/or IFNγ *in vitro*. Each lane was loaded with 5 μg total protein. Results represent three separate experiments.

Figure 4. PGHS-1 and PGHS-2 levels in bone marrow MØ that were isolated from IL-$10^{-/-}$, WT (C57Bl/6), and Balb/c mice and primed with LPS and/or IFNγ *in vitro*. Each lane was loaded with 5 μg total protein. Results represent three separate experiments.

Figure 1.


Spleen
PGE2 (ng/ml)/5 x 10^6 cells
10
8
6
4
2
0
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Medium
IFNγ
LPS
LPS/IFNγ
IL-10-KO
C57Bl/6
Balb/c
Bone marrow
PGE2 (ng/ml)/5 x 10^6 cells
160
120
80
40
0
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#
**
Medium
IFNγ
LPS
LPS/IFNγ
IL-10-KO
C57Bl/6
Balb/c

Figure 2.

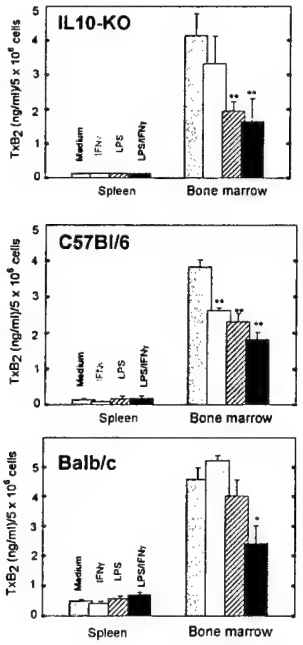

IL10-KO
C57Bl/6
Balb/c
TxB2 (ng/ml)/5 x 10^6 cells
Medium
IFNγ
LPS
LPS/IFNγ
Spleen
Bone marrow

Figure 3.

Splenic MØ

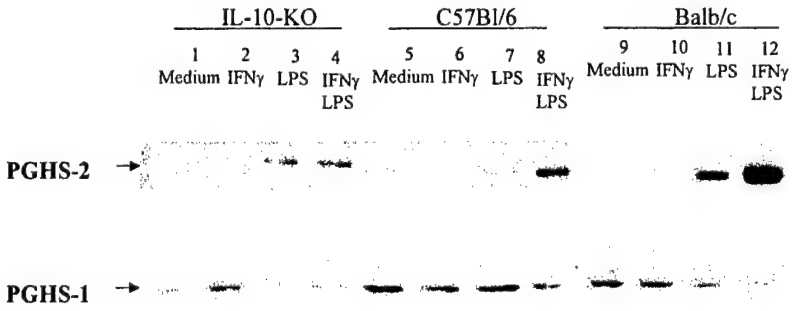
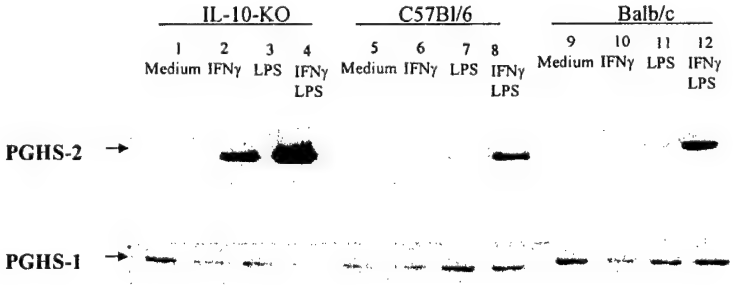


Figure 4.

Bone Marrow MØ



Abstract - International Symposium on Atherosclerosis (ISA) 28403

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Abstract title:

Infections in atherosclerosis --- Mechanisms of Th1-to-Th2 shifts of HSP65 responses

Abstract text (body):

When infected by *Chlamydia pneumoniae* and mycobacteria, immunocompetent hosts initially develop protective Th1 responses against HSP65-producing bacteria. In contrast, patients with atherosclerosis commonly express high antibody titers against mycobacterial HSP65, developing a non-protective Th2 response. Since the antibodies potentially attack heart/arteries through antigenic mimicry, the mechanisms of Th1-to-Th2 shifts are associated with atherogenesis but remain to be elucidated. We found that 1 mg, but not 0.01 mg, heat-killed *M. bovis* BCG given ip to mice induced splenic PGE_2-releasing macrophages (PGE_2-MP) that are considered to induce a Th1-to-Th2 shift. In fact, BCG at 0.01 mg induced Th1 responses against HSP65, whereas BCG at 1 mg induced antibodies against HSP65 after 2 weeks exposure. To determine if similar responses occur in $apoE^{-/-}$ mice, a model for atherosclerosis, BCG at 0, 0.01 or 1 mg/dose were given to 12 week-old $apoE^{-/-}$ mice. PGE_2-MP expressed PGHS-1 and -2 and released PGE_2 *ex vivo*, 14 days after BCG treatments. Compared to WT mice, elevated levels of PGE_2 and serum IgE were detected in control $apoE^{-/-}$ mice. Both PGE_2-MP and serum IgE levels were further increased by the BCG treatments in a dose dependent manner. BCG-treated mice also expressed (i) serum IgG1 and IgG2a antibodies against HSP65 and (ii) Th2 cytokine production *ex vivo* by spleen cells. Finally, treatment with 1 mg of BCG resulted in enhanced development of atherosclerotic lesions compared to the saline treated mice. These results indicate that the $apoE^{-/-}$ mouse is a useful model for further investigations of the mechanisms of Th1-to-Th2 shifts in the development of atherosclerosis. (NIH HL717111 & DOD PR023017).

Topics:

35 Infectious disease

34 Immune-response and inflammation

Presentation Preference:

Poster

Authors of Abstract:

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Appendix V **DAMD17-03-1-0004** **Yoshimi Shibata**

Abstract will be presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington D.C.

Marrow-derived splenic macrophages expressing Cox-2 may contribute to increased PGE_2 production in BCG-immunized mice

Yoshimi Shibata, Mike Smith, Akihito Nishiyama, Hiroyoshi Ohata, Quentin N. Myrvik, Ruth Ann Henriksen

Normal splenic macrophages (MØ) produce <1 ng $PGE_2/10^6$ MØ/ml following ex-vivo stimulation. When mice are immunized with mycobacterial components (HK-BCG, 1 mg/dose, ip), splenic MØ released >10 ng PGE_2/ml at 7-21days and facilitated a Th1-to-Th2 shift of immune responses. To understand the mechanism of splenic PGE_2-MØ formation, we characterized the expression of cellular Cox-2, a key enzyme for PGE_2 synthesis, in splenic MØ isolated from C57Bl/6 mice given HK-BCG ip. We found no Cox-2 expression by splenic MØ on Days 0 (normal), 3 and 4 after HK-BCG treatment. However, biphasic expression of Cox-2 was detected immunohistochemically and by Western blotting on Days 1 and 14. Furthermore, normal splenic MØ incubated with LPS expressed Cox-2 within 1 day. Neither 1-day splenic MØ prepared in vivo by HK-BCG, nor in vitro with LPS released significant amounts of PGE_2. A green fluorescent protein (GFP)-bone marrow chimera was established in C57Bl/6 recipients receiving bone marrow cells iv from GFP-donors. At 14-days after BCG immunization, GFP^+ spleen cells were co-localized with $Cox\text{-}2^+$ cells. We conclude that the presence of Cox-2 alone is not sufficient to induce PGE_2 production by the splenic MØ and that bone-marrow derived MØ may contribute to the increased PGE_2 production observed at 14 days after BCG immunization. (Supported by NIH HL 71711 & DOD PR023017)

Appendix VI **DAMD17-03-1-0004** **Yoshimi Shibata**

Abstract will be presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington D.C.

Mycobacteria-induced osteoclastogenesis and PGE2-releasing macrophage formation in the mouse spleen.

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Atherogenic lesions are often calcified possibly in association with bone resorption that would (is or may?) be accelerated by bacterial infections. The development of osteoclasts (OC) is regulated in bone marrow by osteoblasts and osteotropic factors such as PGE2. However, the events leading to extramedulary osteoclastogenesis are largely unknown. In this study, we determined if BCG induces splenic hemopoiesis including OC and PGE2-releasing macrophages (PGE2-MØ).. Spleen cells were isolated from female Balb/c mice following immunization with heat-killed *Mycobacterium bovis* (BCG) at 1 mg/dose ip. The expression of the calcitonin receptor (CT-R), an 80 Kd OC antigen , was determined cytometrically and by Western blotting using anti-CT-R antibodies (Santa Cruz Biotech). PGE_2 levels released by splenic MØ *ex vivo* were measured by ELISA. We found that the levels of CT-R^+ cells and PGE_2-MØ were increased at 5 days and reached to their peak along with increased hemopoiesis at one week. The levels declined slightly 3 weeks after BCG immunization. Our results indicate a temporal association of the two cell types in BCG-induced splenic hemopoiesis. It still remains to be investigated whether splenic osteoclastogenesis directly contributes to calcification of atherogenic lesions. (Supported by NIH HL 71711 & DOD PR023017)

Abstract will be presented at the 2004 Experimental Biology, 4/17-4/21/04, Washington D.C.

Mechanism of phagocytosed particle-induced IL-12 production in macrophage

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Chitin (N-acetyl-D-glucosamine polymer) can serve as a Th1 adjuvant, thereby limiting Th2 responses. Previously, we found that macrophages (MØ) phagocytose 1-10 μm chitin particles through the mannose receptor, resulting in the production of IL-12, a Th1 cytokine, within 24 h. However, neither soluble chitin nor larger non-phagocytosable chitin particles (>50 μm) induce IL-12 production. To understand the signal-transduction pathways involved in 1–10 μm chitin-induced IL-12 production, we examined the effects of SB203580 (p38 MAPK inhibitor), herbimycin A (PTK inhibitor) and cytochalasin D (actin polymerization inhibitor) on IL-12 production. Splenic MØ isolated from IL-10-KO mice were used to avoid the effect of IL-10, an inhibitor of IL-12 synthesis. LPS was used as a positive control since it induces IL-12 production via p38 MAPK activation. SB203580 at 0.2 μM and 0.3 μM inhibited chitin- and LPS- induced IL-12 production at 50%, respectively. Western blotting showed that both chitin- and LPS-induced p38 MAPK phosphorylation within 20 min. Only chitin-induced IL-12 production was inhibited by 0.1 μM herbimycin A or 0.039 μM cytochalasin D .
Our results indicate that, in the absence of IL-10, IL-12 production in response to either phagocytosis of chitin or stimulation by LPS is dependent at least in part on p38 MAPK activation. However, upstream events diverge such that chitin stimulation, but not LPS stimulation of Il-12 production, depends on an herbimycin A inhibitable PTK and actin polymerization. (Supported by NIH HL71711 and DOD PR023017)

ORAL ADMINISTRATION OF N-ACETYL-D-GLUCOSAMINE POLYMER PARTICLES DOWN-REGULATES ALLERGIC RESPONSES

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BACKGROUND/PURPOSE: Children in military families are at high risk of asthma, and new strategies for reducing the incidence and health care costs are needed. Chitin is a naturally occurring N-acetyl-D-glucosamine polymer and new class of Th1 adjuvant that stimulates IL-12 production by macrophages *in vitro*. This study demonstrates that chitin down-regulates the allergic response in a murine model of allergic asthma. **METHODS**: Ragweed-sensitized BALB/c mice were treated orally with saline or 1- 10 μm chitin particles (8 mg/day for 3 days before and 13 days during ragweed allergen immunization, 7 mice per group). The mice were challenged with ragweed intratracheally on day 11. Three days after the challenge, serum IgE levels and lung eosinophil numbers were quantified. Th2 responses were further explored by measuring cytokine production by spleen cells isolated from the ragweed-immunized mice (controls) and cultured in the presence of ragweed and/or chitin for 3 days. **RESULTS:** The ragweed-sensitized mice treated with saline showed high levels of serum IgE and lung eosinophils, and splenocytes from these animals produced IL-4, IL-5, and IL-10 in vitro. Chitin treatment resulted in a significant reduction of these Th2 parameters ($p<0.01$). **CONCLUSIONS:** Collectively, these results indicate that chitin, which induces innate immune responses, down-regulates Th2-facilitated IgE production and lung eosinophilia in the allergic mouse. Oral administration of chitin therefore represents a potentially effective treatment for IgE-mediated allergic diseases, including childhood asthma. Results of this study will support phase I trials on the effects of oral chitin on childhood asthma.

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